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Regulation of the NF-κB Precursor relish by the *Drosophila* I-κB Kinase Complex: A Dissertation

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REGULATION OF THE NF-κB PRECURSOR RELISH BY THE *Drosophila* IκB KINASE COMPLEX

A Dissertation Presented

By

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”We’re in a race against ourselves, my love.

Either we’ll bring life to dead stars,

or death will descend on our earth.”

Nazım Hikmet Ran

16 March 1958

(Translated from Turkish by Randy Blasing and Mutlu Konuk)

This work is dedicated to my uncle...

Turan Ertürk

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- **Ertürk-Hasdemir D**, Broemer M, Leulier F, Lane WS, Stöven S, Meier P, and Silverman N. The *Drosophila* IκB Kinase Controls Transcription of Antimicrobial Peptide Genes by Direct Phosphorylation of Relish (2008) (submitted)

- Aggarwal K, Rus F, Vriesema-Magnuson C, **Ertürk-Hasdemir D**, Paquette N, and Silverman N. Rudra Interrupts Receptor Signaling Complexes to Negatively Regulate the IMD Pathway. PloS Pathogens (2008) (in press)

- **Deniz Ertürk-Hasdemir**, Nicholas Paquette, Kamna Aggarwal and Neal Silverman (2007). Bug Versus Bug: Humoral Immune Responses in *Drosophila melanogaster*, In Innate Immunity of Plants, Animals, and Humans, Holger Heine, ed. (Springer Berlin Heidelberg), pp. 43-72.

- Kaneko T, Yano T, Aggarwal K, Lim JH, Ueda K, Oshima Y, Peach C, **Ertürk-Hasdemir D**, Goldman WE, Oh BH, Kurata S, Silverman N. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol. 2006 Jul;7(7):715-23.

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ABSTRACT

The innate immune system is the first line of defense against infectious agents. It is essential for protection against pathogens and stimulation of long-term adaptive immune responses. Therefore, deciphering the mechanisms of the innate immune system is crucial for understanding the integrated systems of host defense against microbial infections, which is conserved from insects to humans.

Despite lacking a conventional adaptive immune system, insects can mount a robust immune response against a wide array of microbial pathogens. These innate immune mechanisms have been widely studied in *Drosophila melanogaster*, because of the model system's powerful genetic, genomic and molecular tools. The *Drosophila* immunity relies on cellular and humoral innate immune responses to fight pathogens. The hallmark of the *Drosophila* humoral immune response is the rapid induction of antimicrobial peptide genes in the fat body, the homolog of the mammalian liver. Expression of these antimicrobial peptide genes is controlled by two distinct immune signaling pathways, the Toll pathway and the IMD (immune deficiency) pathway.

The Toll pathway is activated by fungal and Gram-positive bacterial infections, whereas the IMD pathway responds to Gram-negative bacteria. Both pathways culminate in activation of the Rel/NF-κB transcription factors DIF (Dorsal-related immunity factor), Dorsal and Relish, which in turn translocate to the nucleus to induce the antimicrobial peptide genes. DIF and Dorsal are activated by the Toll pathway and control induction of antimicrobial peptide genes such as *Drosomycin*. The NF-κB precursor Relish, which is composed of an N-terminal Rel homology domain and a C-

terminal IκB-like domain, is activated by the IMD pathway and initiates transcription of antimicrobial peptide genes such as *Diptericin*. Although many components of the *Drosophila* immune signaling pathways have been identified, the detailed mechanisms of signal transduction and the molecular interactions between pathway components is mostly unclear.

The IMD pathway is initiated upon recognition of Gram-negative bacterial peptidoglycan (PGN) by the receptor PRRP-LC. The signaling cascade, triggered by this recognition leads to endoproteolytic cleavage and nuclear translocation of the NF-κB module of Relish, while the C-terminal IκB module remains in the cytoplasm. It is known that signal-dependent cleavage and nuclear translocation of Relish requires the *Drosophila* IκB Kinase (IKK) complex and the *Drosophila* caspase-8 homolog DREDD (Death related ced-3/Nedd2-like protein), and that the activated IKK complex can directly phosphorylate Relish. However, the mechanism of IKK-mediated Relish activation has not been fully understood.

My studies have focused on understanding the molecular mechanisms of Relish activation and its regulation by the *Drosophila* IκB Kinase complex. These studies have revealed that the IKK complex controls Relish activation by at least two distinct mechanisms. First, I have shown that the cleavage of Relish requires its interaction with *Drosophila* IKKβ. A predicted death domain fold in the C-terminus of Relish mediates this interaction. Additionally, two serine residues (528 and 529) on Relish that are phosphorylated by the IKK complex following immune stimulation were identified. These phosphorylation sites are not required for immune-induced Relish cleavage. The caspase DREDD can directly cleave unphosphorylated Relish *in vitro* and overexpression

of DREDD leads to Relish cleavage independent of its phosphorylation. The phosphorylations on serines 528 and 529 are also not required for nuclear translocation and DNA binding of Relish. Instead, they are critical for efficient recruitment of RNA Polymerase II to promoters of antimicrobial peptide genes.

My thesis work has shown that Relish interacts with IKKβ through its C-terminal death domain, which is required for its phosphorylation and signal dependent cleavage. The caspase DREDD can directly cleave Relish and cleavage of Relish does not seem to require phosphorylation. On the other hand, IKK-mediated phosphorylation of Relish is required for efficient recruitment of RNA Polymerase II to promoters of antimicrobial peptide genes. Together these data describe a novel death domain in Relish and provide insights into the molecular mechanism of IKKβ mediated Relish activation.

CHAPTER I:

INTRODUCTION

Insects are exposed to various pathogens in their natural environment. Therefore, they have developed sophisticated mechanisms to recognize and fight infectious microorganisms. These mechanisms are best characterized in *Drosophila melanogaster*, which is a potent model for studying mechanisms and evolution of insect immunity as well as shared strategies with other organisms. Striking similarities in addition to major differences to mammalian innate immunity, genomic sequencing of *Drosophila melanogaster* and 11 other *Drosophila* species, and the presence of powerful genetic and molecular tools makes *Drosophila* a favored model system (Brennan and Anderson, 2004; Cherry and Silverman, 2006; Hultmark, 2003; Lemaitre and Hoffmann, 2007).

The immune response against microbes in *Drosophila* is a multi-layered system. Structural barriers such as a chitin-based exoskeleton and the endothelia form the first layer of defense. Once a pathogen has breached those layers, other immune mechanisms are activated. Recognition is the first step in a cascade of events that leads to an immune response. Pattern recognition receptors recognize microbial products, such as peptidoglycan (PGN). Upon recognition, appropriate immune responses are initiated, including phagocytosis, melanization, encapsulation, and coagulation. *Drosophila* also release cytotoxic molecules and reactive oxygen species, produce other defense molecules such as lysozyme, and proteolytic and hydrolytic enzymes. The final layer and the hallmark of *Drosophila* immune response is the systemic production of antimicrobial peptides in the fat body, which effectively kill the microbes.

These layers of defense are also classified as cellular and humoral responses. Various cellular responses are regulated by hemocytes. For example, phagocytosis is mediated by plasmatocytes and encapsulation by lamellocytes, while melanization

requires crystal cells. Humoral responses depend on antimicrobial peptides, which are produced by the fat body cells and released into the hemolymph.

At least two different pathways regulate the production of antimicrobial peptides in *Drosophila*. The Toll pathway is stimulated by fungal and Gram-positive bacterial infections, while the IMD (immune deficiency) pathway is triggered by Gram-negative bacteria.

1.1 The History

The discovery of the causative agent of pébrine disease and it's diagnosis in silkworms by Louis Pasteur in 1865, was a huge development for the silk industry and the beginning of the rapidly growing insect immunity field (Brey, 1998). The role of arthropods as hosts and vectors of microbes causing plant, animal and human diseases such as yellow fever (Chaves-Carballo, 2005) led researchers to a number of studies in the late 1800s and early 1900s to understand extracellular and intracellular flora of various insects (Steinhaus, 1940).

By the end of 1960s, it was already known that pathogens such as fungi, protozoa, viruses, and bacteria could infect insects. In response to these infections, insects activated cellular and humoral immune defenses, including phagocytosis and the production of antimicrobial substances (Heimpel and Harshbarger, 1965). A milestone in the insect immunity field was the study by Hans Boman and colleagues on the inducible antibacterial defense mechanisms of *Drosophila* (Boman et al., 1972). In subsequent years, a number of studies were done to characterize specific antimicrobial peptides (AMP) and the genes encoding them in various insects, including *Drosophila* (Hultmark

et al., 1983; Kylsten et al., 1990; Samakovlis et al., 1990; Steiner et al., 1981; Sun et al., 1991).These antimicrobial peptides are small, cationic molecules that are effective against specific classes of pathogens.

After the discovery of the transcription factor NF-κB in mammals in 1986, promoters of the antimicrobial peptide genes in insects were also found to have κB sequences, indicating that they are transcriptionally regulated by NF-kB-like transcription factors (Engstrom et al., 1993; Kappler et al., 1993).

In the following years, a great number of studies were done to understand the recognition of microbes and the regulation mechanisms of signaling cascades that lead to NF-κB-dependent gene expression of antimicrobial peptides.

1.2 Overview of the *Drosophila* Immune Response

Drosophila has a multi-layered system for host defense. The chitin-based exoskeleton and chitinous internal structures form a physical barrier. If a pathogen breaches these barriers, several immune effector mechanisms respond, including cellular responses (i.e. phagocytosis, encapsulation, melanization) and humoral responses (i.e. antimicrobial peptides). Antimicrobial peptides are found both locally, at the site of infections, and systemically in the insect sera, or hemolymph. In terms of the systemic humoral response, the fat body is the major site of antimicrobial peptide production, although other tissues also contribute, including the malphigian tubules and circulating blood cells, known as hemocytes. The local response induces antimicrobial peptide gene expression in epithelial tissues, like the trachea and the gut (Ferrandon et al., 1998; Liehl et al., 2006; Muyskens and Guillemin, 2008; Silverman and Paquette, 2008; Tzou et al.,

2000). Recognition is the first step in a cascade of events that leads to these immune responses. Microbial products, often cell wall components, are detected by recognition receptors, which in turn stimulate signaling pathways that culminate in the induction of antimicrobial peptide gene expression (Aggrawal and Silverman, 2007; Ferrandon et al., 2007). The antimicrobial peptides are so critical for resistance to infection, that transgenic expression of a single antimicrobial peptide can protect immunodeficient flies (Tzou et al., 2002). These antimicrobial peptides are effective against different classes of pathogens. For example, Defensin acts against Gram-positive bacteria (Dimarcq et al., 1994), and Diptericin, Drosocin and Attacin are effective against Gram-negative bacteria (Asling et al., 1995; Bulet et al., 1993; Charlet et al., 1996; Reichhart et al., 1992; Wicker et al., 1990), whereas Drosomycin shows anti-fungal activity (Landon et al., 1997; Michaut et al., 1996). Metchnikowin and Cecropin are both anti-bacterial and anti-fungal (Ekengren and Hultmark, 1999; Levashina et al., 1995; Samakovlis et al., 1990).

At least two different pathways regulate the expression of antimicrobial peptide genes in *Drosophila*. The Toll pathway is stimulated by fungal and many Gram-positive bacterial pathogens. In contrast, the immune deficiency (IMD) pathway is triggered by Gram-negative bacteria.

1.3 The Toll Pathway

The Toll pathway responds to Gram-positive bacterial and fungal infections (Lemaitre et al., 1996). Unlike human Toll-like receptors (TLRs) *Drosophila* Toll does not directly bind pathogens or microbe-derived compounds (Weber et al., 2003). Instead, Toll is a cytokine receptor, activated by the serum protein Spätzle. Spätzle is produced as

a pro-protein, with a disulfide-linked dimeric structure. In order to activate the Toll pathway, pathogens activate serine protease cascades that culminate in Spätzle cleavage, liberating the mature Toll ligand (Denton and Morisato, 1998; Hu et al., 2004; Jang et al., 2006; Weber et al., 2003).

Recognition of Gram-positive bacteria involves the receptors PGRP-SA and PGRP-SD (Bischoff et al., 2004; Gobert et al., 2003; Michel et al., 2001; Pili-Floury et al., 2004). In addition, PGRP-SA functions in a complex with Gram-negative binding protein 1 (GNBP-1), which is a PGN processing enzyme. Both the receptors PGRP-SA and PGRP-SD recognize lysine-type PGN, but probably with slightly different specificities. Recognition of the *Micrococcus luteus* requires the PGRP-SA/GNBP-1 complex. Flies lacking either of these receptors fail to induce antimicrobial peptide gene expression following *M. luteus* infection and are highly susceptible to this microbe (Michel et al., 2001). In contrast, Gram-positive bacteria like *Staphylococcus aureus*, *Streptococcus pyogens*, *Staphylococcus saprophyticus*, and *Enterococcus faecalis* are recognized by either PGRP-SA or PGRP-SD and only the double *PGRP-SA*, *PGRP-SD* mutant is susceptible to infection with these bacteria (Bischoff et al., 2004). These results suggest that PGRP-SD recognizes a peptidoglycan-derived structure found in *Staphylococcus aureus*, *Streptococcus pyogens*, *Staphylococcus saprophyticus* and *E. faecalis* but not in *M. luteus*.

The exact function of GNBP1 in the recognition of lysine-type PGN is still under active investigation. GNBP-1 recognizes certain lysine-type PGN (e.g. *M. luteus* but not *Sta. aureus* PGN), and cleaves it into smaller muropeptides (Filipe et al., 2005; Wang et al., 2006). The minimal structure that activates the Toll pathway is a muropeptide dimer,

composed of two disaccharide-tetrapeptides cross-linked via a penta-peptide bridge. In fact, GNBP1 generates these small active PGN fragments from Polymeric *M. luteus* PGN. But, GNBP1 is also capable of digesting PGN fragments even further, into monomers, which are inactive. The interaction between GNBP1 and PGRP-SA is enhanced by the presence of hydrolyzed PGN fragments. Another recent report demonstrated *in vitro* digestion of *Sta. aureus* PGN into lysozyme-generated PGN fragments that activate the Toll and phenoloxidase pathways (in *Drosophila* and *Tenebrio*, respectively (Park et al., 2007b); suggesting that other enzymes (in addition to GNBP1) are capable of processing various lysine-type PGN for presentation to PGRP-SA. *Drosophila* encodes eight lysozyme homologs as well as five GNBP-related genes; perhaps some of these are involved in processing *Staphylococcus aureus* PGN.

Detection of fungal infections relies on two sensor systems that are partially redundant. Fungal Polysaccharides are recognized by the receptor GNBP-3 (Gottar et al., 2006). *In vitro*, GNBP3 binds the yeast *Candida albicans* as well as curdlan, an insoluble Polymer of β-(1,3)-glucan, which is present in the yeast cell wall, but not bacterial PGN. A second pathway, which requires a serine protease known as persephone (PSH), defines a second fungal recognition pathway. Live, entomopathogenic molds, such as *B. bassiana* and *M. anisopliae* stimulate the PSH pathway in addition to the GNBP3-dependent pathway, while yeast or killed molds activate only the GNBP3-dependent pathway. Instead of relying on a pattern recognition receptor, the PSH-dependent pathway is probably stimulated directly by pathogen-produced proteases, such as PR1A, which are released by pathogenic fungi to breakthrough the host cuticle. These results suggest that

the *Drosophila* innate immune system can directly recognize virulence factors, analogous to the plant defense system.

Once activated, PGRP-SA/GNBP1, PGRP-SD, GNBP3 or PSH leads to Spätzle cleavage by activating serine protease cascades. During embryonic development a cascade of CLIP domain serine proteases leads to Spätzle activation. Mutants for these proteases (*snake*, *easter*) mount a wild-type immune response, indicating that these proteases are not required for the immune response (Lemaitre et al., 1996). A genetic screen led to the identification of PSH, which is homologous to Snake and is required for the cleavage of Spätzle in response to entomopathogenic fungal infections (Ligoxygakis et al., 2002). The *psh* mutants were first discovered as suppressors of the *necrotic* (*nec*) phenotype. *nec* encodes a serine protease inhibitor of the serpin family and lack of *nec* leads to constitutive activation of the Toll pathway in a *psh*-dependent manner (Levashina et al., 1999; Ligoxygakis et al., 2002). Another serine protease, Grass, is required only for the resistance to Gram-positive bacterial infection (Kambris et al., 2006). Recent studies showed that all these protease pathways appear to converge on two chymotrypsin-like serine proteases: Spirit and the Spätzle-processing enzyme (SPE). Spirit is thought to be the protease that cleaves and activates SPE, although this has not been directly demonstrated, while SPE directly cleaves pro-Spätzle, releasing the active C106 fragment. Both *Spirit* and *SPE* are required to resist both Gram-positive and fungal infections (Jang et al., 2006; Kambris et al., 2006).

Spätzle binding induces dimerization of the Toll receptor. Although the ligand is a symmetric dimer, biophysical studies indicate that the Spätzle-induced Toll dimer is asymmetrical (Weber et al., 2003). It is not yet clear whether the asymmetric aspect of

the ligand-induced Toll dimer is critical for the activation of intracellular signaling. Dimerization of the Toll receptor is believed to recruit a pre-existing Myd88/Tube complex (Figure1.2). Furthermore this complex associates with the kinase Pelle (the homolog of mammalian IRAK) (Sun et al., 2002a). The assembly of the resulting receptor complex occurs via two distinct functional domains. While the interaction between Toll and Myd88 occurs via their Toll/IL-1R (TIR) domains, Myd88, Tube, and Pelle interact in a trimeric complex via death domains (DD) found in each protein (Sun et al., 2002a; Sun et al., 2002b; Tauszig-Delamasure et al., 2002; Towb et al., 1998). Although the death domains of these proteins are necessary for their interactions, Myd88 and Pelle do not interact directly; Tube acts as the core of the trimeric complex (Sun et al., 2002a). Thus the activated Toll receptor interacts directly with Myd88, which interacts with Tube, which ultimately recruits the kinase Pelle. Similar IRAK-kinase recruitment via an adapter complex is seen in mammalian Myd88-dependent TLR signaling.

Drosophila TNF-receptor-associated factor 2 (dTRAF2), the homolog of mammalian TRAF6, may also play a role in Toll signaling; however its role is unclear. In transiently transfected *Drosophila* cells, Pelle interacts with dTRAF2 and co-expression of Pelle and dTRAF2 synergistically activates the Toll pathway target gene *Drosomycin* (Shen et al., 2001). However RNAi to *dTraf2* shows no suppression of antimicrobial peptide gene expression after stimulation of the Toll or IMD pathways (Sun et al., 2002a; Zhou et al., 2005). In adult flies, overexpression of dTRAF2 is able to induce antimicrobial peptide gene expression and nuclear translocation of DIF as well as Relish. Interestingly, *dTraf2* null larvae exhibited reduced, but not abolished, levels of

antimicrobial peptide gene expression following *Escherichia coli* infection (Cha et al., 2003). These data suggested that dTRAF2 may function in both the IMD and Toll pathways, but bypass mechanisms may be present which circumvent dTRAF2 in both cases.

Infections by Gram-positive bacteria and fungi culminate in the nuclear translocation of NF-κB proteins DIF and/or Dorsal. DIF is the main regulator of Toll signaling in both adults and larvae, whereas Dorsal is specifically required for the immune response in larvae. Dorsal was first identified for its role in dorso-ventral patterning in the developing embryo (Santamaria and Nusslein-Volhard, 1983). The intracellular signaling components that lead to activation of Dorsal are the same in both the early embryo and in the immune response (Drier and Steward, 1997). DIF/Dorsal is sequestered in the cytoplasm by its interaction with the IκB protein Cactus. The six-ankyrin repeats of Cactus are required for this interaction. In the embryo, Cactus and Dorsal are found in a complex of dorsal homodimer interacting with one molecule of Cactus (Isoda and Nusslein-Volhard, 1994). Upon signaling, Cactus is degraded and DIF or Dorsal translocates to the nucleus (Belvin et al., 1995; Bergmann et al., 1996; Gillespie and Wasserman, 1994; Reach et al., 1996; Wu and Anderson, 1998). Cactus degradation, like IκBs, is controlled by phosphorylation and ubiquitin/protesome-mediated degradation. Initially, serines 74, 78, 82, and 83, in a region similar to the IκBα phosphorylation site, were thought to regulate signal-dependent degradation of Cactus (Bergmann et al., 1996; Reach et al., 1996). In contrast, later studies found that the N-terminal 125 amino acids are critical for signal-induced Cactus degradation, but the IκBα-like target motif between residues 74 and 83 is dispensable for degradation (Fernandez et

al., 2001). Instead, Fernandez et al. (2001) identified another IκBα-like target motif around serine 116 that is sufficient for degradation. Serines 74, 78, and 116 must all be changed to alanine to block Cactus degradation in the embryo. In addition, phosphorylation of the PEST domain, found at the C-terminus of Cactus, is implicated in its signal-independent degradation (Liu et al., 1997). However, neither of the two *Drosophila* IKK-related kinases (IKKε, IKKβ) is required for Toll-mediated Cactus phosphorylation and degradation. Although *Drosophila* IKKβ can phosphorylate Cactus *in vitro* (Kim et al., 2000), it is not required for *Drosomycin* expression in cells or in flies (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000). Although the sequence motifs that are phosphorylated are very similar to those critical for IκBα phosphorylation in human cells, the kinase that phosphorylates Cactus is yet to be identified. Once phosphorylated, Cactus is likely ubiquitinated via the Slimb-SCF E3-ligase complex. *Drosophila* embryos mutant for *slimb*, the βTrCP homolog, are unable to activate the Dorsal target genes *twist* and *snail* (Spencer et al., 1999). Interestingly, Cactus degradation is required but not sufficient for efficient nuclear translocation of Dorsal during development (Bergmann et al., 1996).

Degradation of Cactus and nuclear translocation of DIF (and Dorsal) leads directly to the transcriptional induction of many immune responsive genes (De Gregorio et al., 2001; De Gregorio et al., 2002; Irving et al., 2001). For example, the well characterized AMP genes *Defensin*, *Drosomycin*, *Cecropin* and *Metchnikowin* are activated by Toll signaling. The promoter/enhancer regions of all these AMP genes include κB-sites where DIF or Dorsal bind (Senger et al., 2004). In addition, Toll signaling leads to the activation of other less well characterized genes, some of which

may be AMPs while others may control different facets of the immune response. In fact, Toll signaling is linked to the activation of the cellular immune response and the proliferation of hemocytes (Qiu et al., 1998; Zettervall et al., 2004). Also, many components of the Toll pathway are regulated by Toll signaling (De Gregorio et al., 2001; Lemaitre et al., 1996). Most notably, Cactus is up-regulated in response to immune challenge via the Toll pathway. This generates a negative feedback loop to down-modulate the cascade (Nicolas et al., 1998).

Coactivators that function with *Drosophila* NF-κB proteins have not been extensively studied. One study reported that dTRAP80 is required for DIF-induced transcriptional activation of *Drosomycin* in S2 cells (Park et al., 2003). Also, Helicase89B, a SNF2-like ATPase, is involved in activation of antimicrobial peptides in both the Toll and IMD pathways, and is thought to link NF-κB factors to the basal transcription machinery (Yagi and Ip, 2005). Another study demonstrated that *Drosophila* CBP is a coactivator for Dorsal, and the Dorsal-dependent activation of *twist* requires *nejire*, the CBP encoding gene (Akimaru et al., 1997).

Post-translational modifications are major regulators of transcription factors. Both NF-κB and IκB proteins are subject to various modifications. For example, in embryos it was demonstrated that Dorsal is multiply and dynamically phosphorylated during Toll signaling (Gillespie and Wasserman, 1994). Phosphorylation of serine 312 is implicated in Dorsal stability, and phosphorylation of serine 317 is critical for optimal nuclear translocation of Dorsal in the embryo (Drier et al., 1999). The kinases responsible for these modifications are not known yet. One candidate might be the *Drosophila* atypical protein kinase C (ζPKC), which in cell culture is required for the Toll-signaling pathway

but does not affect Cactus degradation. ζPKC can phosphorylate DIF *in vitro* (Avila et al., 2002). The nature and function of this phosphorylation event has yet to be identified.

The Toll and IMD pathways are thought to be activated independently and initiate specific responses to different microorganisms. However, some AMPs are activated by both Toll and IMD pathways. Tanji et al. recently showed that some antimicrobial peptide genes have distinct κB elements in their enhancer region (e.g. *Drosomycin*) that respond to either Relish or DIF, and optimal gene induction occurs only when both the Toll (DIF) and IMD (Relish) pathways are activated, suggesting synergistic regulation of AMPs by two pathways (Tanji et al., 2007).

1.4 The IMD Pathway

The IMD pathway is potently activated by DAP-type PGN derived from Gram-negative bacteria and certain Gram-positive bacteria, such as *Bacillus* spp. Initially, it was believed that LPS activated the IMD pathway (Silverman et al., 2000; Werner et al., 2003). However, this did not account for the activation of the IMD pathway by certain Gram-positive bacteria (Kaneko et al., 2004; Lemaitre et al., 1997; Leulier et al., 2003). Subsequently, it was demonstrated that the commercial LPS preparations often used to stimulate the IMD pathway, in animals or cell lines, are contaminated with PGN, and it is this PGN that activates the IMD pathway (Kaneko et al., 2004; Leulier et al., 2003; Werner et al., 2003). Highly purified, PGN-free LPS does not stimulate IMD signaling in flies or fly cells.

Recognition of DAP-type PGN involves the receptors PGRP-LC and PGRP-LE (Choe et al., 2002; Gottar et al., 2002; Leulier et al., 2003; Ramet et al., 2002; Takehana et al., 2002). *PGRP-LC* encodes three alternatively spliced transcripts *PGRP-LCa*, *-LCx*,

-*LCy*. All three isoforms encode single-pass transmembrane cell surface receptors. They each have distinct extracellular domains, which include a PGRP motif, anchored to the identical transmembrane and cytoplasmic domains (Werner et al., 2000). PGRP-LE encodes only one protein, which lacks both a signal sequence and a transmembrane domain. Although *PGRP-LC* null flies, which lack all three isoforms, induce dramatically reduced levels of antimicrobial peptide gene expression following infection with Gram-negative bacteria, such as *Escherichia coli* and *Agrobacterium tumefaciens*, they are not particularly susceptible to infection with all Gram-negative bacteria. For example, *PGRP-LC* mutants are sensitive to *A. tumefaciens*, *Erwinia carotovora carotovora*, and *Enterobacter cloacae*, but not *E. coli* and *B. megaterium* (Choe et al., 2005; Gottar et al., 2002; Takehana et al., 2004). In contrast, mutants that abolish signaling through the IMD pathway, such as null alleles in IKK genes (see below), are highly susceptible to all Gram-negative bacteria. Therefore, it was hypothesized that another receptor must also recognize and respond to Gram-negative bacteria. Moreover, it was suggested that relatively low levels of antimicrobial peptide gene induction, as observed in *PGRP-LC* mutants, are sufficient to protect against infection with many Gram-negative bacteria. Genetic experiments suggested that PGRP-LE is the alternate receptor for the IMD pathway. Double *PGRP-LC*, *PGRP-LE* mutants are hypersusceptible to most Gram-negative bacteria, similar to other null mutants in the IMD pathway, and these double mutants do not induce detectable levels of antimicrobial peptide genes following infection. Overexpression of either PGRP-LC or PGRP-LE, in flies or in cell culture, is sufficient to drive AMP expression through the IMD pathway. PGRP-LE overexpression also activates the phenoloxidase cascade (Park et al., 2007b).

Why might flies have two receptor systems (or perhaps four, if one considers the three *PGRP-LC* splice isoforms) for the recognition of bacteria and the activation of the IMD pathway? It appears that these receptors serve to recognize different forms of DAP-type PGN and to protect distinct niches. Monomeric and Polymeric forms of DAP-type PGN are recognized by different receptors. In cell culture and in flies, only PGRP-LCx is required for recognizing Polymeric PGN (isolated from *E. coli*). In contrast, both PGRP-LCx and -LCa are required in cultured cells for recognition of the monomeric fragment of DAP-type PGN known as TCT (Kaneko et al., 2004; Stenbak et al., 2004).The role of PGRP-LCy in microbial recognition is still unknown.

TCT binds PGRP-LCx directly, and then this ligand/receptor complex interacts with PGRP-LCa (Chang et al., 2005; Mellroth et al., 2003). The crystal structure of TCT bound to the ectodomains of PGRP-LCx and -LCa has been solved. TCT binds in the deep PGN binding cleft of PGRP-LCx, typical of PGRP--muropeptide interactions. The disaccharide unit of TCT makes important contributions to the interactions between PGRP-LCx (bound to TCT) and PGRP-LCa (Chang et al., 2006).

In adult flies, the recognition of monomeric TCT is even more complex. *PGRP-LC* null flies induce antimicrobial peptide gene expression following injection of TCT, but not after injection of Polymeric *E. coli* PGN. *PGRP-LE* mutants respond normally to both monomeric and Polymeric PGN. However, double *PGRP-LC*, *PGRP-LE* mutants fail to respond to TCT. Thus, in adult flies TCT can be recognized by either PGRP-LC or PGRP-LE. As mentioned previously, PGRP-LE lacks a transmembrane domain and a signal peptide, and is likely an intracellular receptor that recognizes small fragments of PGN-like TCT. These small PGN fragments may be able to gain access to PGRP-LE

within the cell, while larger Polymeric PGNs can only stimulate the cell surface receptor PGRP-LC. In support of this model, overexpression of PGRP-LE in the malphigian tubules (an immune-responsive kidney-like organ) triggers IMD signaling in a cell autonomous manner. Malphigian tubules ex vivo responded to TCT primarily through PGRP-LE and independently of the cell surface receptor PGRP-LC, and PGRP-LE was detected within these cells (Kaneko et al., 2006). The PGRP domain of PGRP-LE binds TCT with a K_d of about 27 nM, and TCT induces the formation of PGRP-LE multimers (Kaneko et al., 2006; Lim et al., 2006). The interactions responsible for TCT-induced PGRP-LE multimerization are very similar, in molecular detail, to those responsible for the TCT-mediated PGRP-LCx/LCa dimer. Because PGRP-LCa cannot bind TCT in a typical PGN binding cleft (Chang et al., 2006; Chang et al., 2005), the LC complex is limited to a dimeric form, while PGRP-LE forms a head-to-tail multimer, with each subunit binding to TCT and interacting with another subunit (Lim et al., 2006).

In addition to its role as intracellular receptor, several findings argue that PGRP-LE also functions outside the cell. When overexpressed in the fat body, PGRP-LE stimulates the IMD pathway in a cell non-autonomous manner. And, the PGRP domain of PGRP-LE (PRGP-LE^{pg}) is found in the hemolymph (the insect sera). It is hypothesized that PRGP-LE^{pg} binds PGN in the hemolymph and presents it to the cell surface receptor PGRP-LC, analogous to CD14/LPS interactions in mammals. Supporting this model, overexpression of PGRP-LE in the fat body induces IMD signaling in a manner that depends in part on PGRP-LC, and expression of PGRP-LE^{pg} in cell culture leads to an enhancement of the PGRP-LC-mediated response to TCT.

Although several lines of evidence strongly suggest that PGRP-LE^{pg} is found in the hemolymph, it is not clear how PGRP-LE is released from cells.

The molecular mechanism by which PGN binding to either PGRP-LC or PGRP-LE leads to activation of the IMD pathway is still unclear. The cytoplasmic domain of PGRP-LC is responsible for initiating this signal transduction cascade (Choe et al., 2005). Epistatic experiments suggest that the *imd* protein functions immediately downstream of PGRP-LC and upstream of all other known members of the pathway. IMD is a death domain protein similar to mammalian receptor interacting protein 1 (RIP1) (Georgel et al., 2001) and immunoprecipitation experiments showed that PGRP-LC and IMD interact (Choe et al., 2005; Kaneko et al., 2006). Kaneko et al. (2006) identified a RIP homotypic interaction motif (RHIM)-like domain that is crucial for signaling by PGRP-LC, when over-expressed or following infection (Kaneko et al., 2006). The RHIM domain, a motif of approximately 35 amino acids, was first identified in mammalian RIP1, RIP3, and in the adaptor protein Trif (Meylan et al., 2004; Sun et al., 2002b). The RHIM domain of Trif interacts with RIP1 and RIP3; and RIP1 and RIP3 also interact with each other through the RHIM domain. The Trif--RIP1 interaction is implicated in TLR3-induced NF-κB activation (Meylan et al., 2004). Likewise, the RHIM-like domain of PGRP-LC is critical for signaling. However, the PGRP-LC RHIM-like domain is not necessary for the interaction between PGRP-LC and IMD. Instead, PGRP-LC interacts with IMD via a region that is not required for signaling (Kaneko et al., 2006). Thus, the PGRP-LC/IMD interaction appears to be superfluous for the activation of the pathway. Although the N-terminal signaling domains of PGRP-LC and PGRP-LE are not homologous, a RHIM-like motif was also identified in PGRP-LE. Mutation of the PGRP-LE RHIM-like motif

blocks the signaling induced by forced expression of this intracellular receptor. The mechanism by which the RHIM-like domains of PGRP-LC and -LE function to transduce IMD signaling remains unclear. Perhaps the RHIM-like domain interacts with some unidentified component of the pathway.

Downstream of PGRP-LC and the *imd* protein, signal transduction through the IMD pathway leads to the *Drosophila* TAK1 homolog and then activation of the *Drosophila* IKK complex (Silverman and Maniatis, 2001; Silverman et al., 2003; Vidal et al., 2001) (Figure1.2). The molecular mechanisms involved in signaling to TAK1 are still unclear, although RNAi-based experiments in cultured cells suggest that ubiquitination may play a key role. Work by Zhou and colleagues indicated that the E2 ubiquitin conjugating enzyme complex of dUEV1A and Bendless (the *Drosophila* Ubc13 homolog) functions downstream of IMD yet upstream of TAK1 in the IMD pathway (Zhou et al., 2005). The mammalian homologs of this E2 complex, Uev1A and Ubc13, are responsible for K63-Polyubiquitination. Unlike K48-Polyubiquitination, which leads to proteasomal degradation, K63-Polyubiquitin chains are often regulatory and are used to recruit and activate other signaling components. Thus, it is highly probable that K63-Polyubiquitination plays an important role in the IMD signaling pathway between IMD and dTAK1. To date however, no K63-Polyubiquinated protein has been identified in the IMD pathway.

Also, the E3 ligase involved in the IMD pathway remains elusive. Recently the *Drosophila* inhibitor of apoptosis protein 2 (dIAP2) was identified as a member of the IMD signaling pathway (Gesellchen et al., 2005; Kleino et al., 2005; Leulier et al., 2006; Valanne et al., 2007). Similar to other E3 proteins, dIAP2 contains a RING domain which

is required for IMD signaling (Huh et al., 2007). Although dIAP2 has not yet been epistatically placed in the IMD signaling cascade, it is a good candidate to act as the E3 ligase, along with the dUEV1A/Bendless E2 complex. In addition, dTRAF2 could function as an E3 in the IMD pathway in some circumstances, as described above in the Toll signaling discussion (Cha et al., 2003).

The apical caspase DREDD is also thought to play a role between IMD and TAK1, perhaps functioning as an E3-ligase accessory factor (Zhou et al., 2005). Together, the E2/E3 complex of dUEV1A, Bendless, dIAP2 (and/or dTRAF2), and perhaps DREDD may mediate the K63-Polyubiquitination of some unidentified member of the IMD pathway. This ubiquitinated protein is likely critical for signaling to TAK1, the next component in the pathway.

TAK1 may function in a complex with the *Drosophila* TAB2 homolog (Zhuang et al., 2006). Similar to mammalian TAB2, which was originally identified as a TAK1 binding protein, *Drosophila* TAB2 contains a conserved K63 Polyubiquitin binding domain (Wang et al., 2001; Zhou et al., 2005), lending more credibility to the notion that ubiqutination plays a crucial role in IMD signaling. Signaling by the TAK1/TAB2 complex leads to the simultaneous induction of two downstream branches of the IMD pathway, which culminate in JNK or NF-κB/Relish activation (Silverman et al., 2003).

The JNK arm of the IMD pathway is activated by TAK1-mediated signaling to Hemipterous, the *Drosophila* MKK7/JNKK homolog (Chen et al., 2002; Holland et al., 1997; Sluss et al., 1996). Hemipterous then goes on to phosphorylate the *basket* protein (JNK), which activates *Drosophila* AP-1. Signaling through the IMD/JNK pathway has been linked to the up-regulation of wound repair and stress response genes (Boutros et

al., 2002; Silverman et al., 2003). Yet, the precise role that JNK signaling plays in the IMD pathway is controversial. Several reports have concluded that JNK signaling is not involved in AMP gene induction. Instead, AMP gene expression relies entirely on the NF-κB/Relish branch of the IMD pathway (Boutros et al., 2002; Silverman et al., 2003). In fact, an unidentified product of the Relish branch of the IMD pathway was proposed to inhibit JNK signaling (Park et al., 2004), while the JNK pathway was proposed to directly inhibit AMP gene expression by recruiting histone deacetylases (Kim et al., 2005). However, Delaney and colleagues (2006) concluded that the TAK1/JNK branch of the IMD pathway is critical for AMP gene induction, at least in clones of JNK-deficient cells within the larval fat body (Delaney et al., 2006). The role of the JNK pathway in antimicrobial gene expression remains controversial and further work will be necessary to clarify whether JNK has a positive and/or, negative role in the process.

In parallel to JNK activation TAK1 is also required for induction of the NF-κB/Relish branch of the IMD pathway, through activation of the *Drosophila* IKK complex (Silverman et al., 2003; Vidal et al., 2001). The *Drosophila* IKK complex contains two subunits: a catalytic kinase subunit encoded by *ird5* (IKKβ) and a regulatory subunit encoded by *kenny* (IKKγ) (Rutschmann et al., 2000; Silverman et al., 2000). In S2 cells, it was clearly demonstrated that the IKK complex is activated rapidly following immune stimulation and this activation requires TAK1 (Silverman and Maniatis, 2001; Silverman et al., 2003). Activated IKK complex can directly phosphorylate Relish, which is then endoproteolytically cleaved in a caspase dependent manner and translocates to the nucleus to activate antimicrobial peptide genes.

1.6 NF-κB Proteins

The Rel/NF-κB proteins are a family of highly conserved transcription factors that control expression of genes involved in innate and adaptive immunity, inflammation, cell proliferation and apoptosis in both mammals and insects (Dutta et al., 2006; Hayden et al., 2006; Karin, 2006; Silverman and Maniatis, 2001). NF-κB proteins share a highly conserved 300-amino-acid N-terminal domain called the Rel-homology domain (RHD) which mediates DNA binding, dimerization and interaction with inhibitory IκB proteins. The RHD may also contain motifs for nuclear localization and transactivation (Perkins et al., 1997; Schmitz et al., 1995). NF-κB proteins are retained in the cytoplasm of unstimulated cells by the inhibitory IκB proteins. In response to immune challenge, IκB proteins are degraded, releasing the NF-κB transcription factors, which then translocate to the nucleus to activate target gene expression. In mammals, NF-κB family members include RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), while *Drosophila* encodes three family members: DIF (Ip et al., 1993; Manfruelli et al., 1999; Meng et al., 1999), Dorsal (Reichhart et al., 1993; Roth et al., 1989; Steward, 1987; Steward et al., 1984) and Relish (Dushay et al., 1996; Hedengren et al., 1999) (Figure 1.1).

DIF and Dorsal are similar to mammalian RelA (p65). They are retained in the cytoplasm by Cactus, the only member of the IκB protein family in *Drosophila* (Geisler et al., 1992; Lemaitre et al., 1996). In contrast, the compound protein Relish resembles mammalian NF-κB precursors p100 and p105 with an N-terminal RHD and a C-terminal IκB-like domain, thus Relish is sequestered in the cytoplasm through this C-terminal

domain. NF-κB transcription factors can regulate gene expression by binding as dimers to these κB sites (Engstrom et al., 1993; Gross et al., 1996). Although the most common NF-κB complex in mammalian cells is a p50--p65 heterodimer, it is likely that the *Drosophila* NF-κB factors preferentially form homodimers (Chen et al., 1998; Engstrom et al., 1993; Tanji et al., 2007; Wirth and Baltimore, 1988).

NF-κB/Rel Precursor Proteins; Relish, NF-κB1 and NF-κB2

Relish is a bipartite protein similar to mammalian NF-κB precursors p50/p105 (NF-κB1), and p52/p100 (NF-κB2). It contains an N-terminal Rel homology domain (RHD) and an inhibitory IκB domain with six ankyrin repeats that holds the protein in the cytoplasm. Upon infection with Gram-negative bacteria, *Relish* expression is strongly induced in adult flies (Dushay et al., 1996). *Relish* mutant flies show extreme sensitivity to infections and fail to induce antimicrobial genes after bacterial infection (Hedengren et al., 1999). Although the *Relish* locus encodes an embryo specific isoform, *Relish* does not seem to have a role in development because homozygous Relish mutants are viable and fertile.

In mammals, both p50 and p52 are synthesized from the precursor proteins p105 and p100 respectively, which contain IκB-like C-termini with ankyrin repeat motifs. In the cytoplasm, p100 and p105 can function like IκB proteins and inhibit the activity of other NF-κB subunits (Perkins, 2007). p100 and p105 are regulated through complete degredation or processing. After processing, p52 and p50 translocate to the nucleus and function as nuclear transcription factors. IKKβ-mediated phosphorylation of the C-terminal region of p105 at Ser923 and Ser927 leads to complete degradation of p105 in a

signal-dependent manner (Heissmeyer et al., 2001). IKKβ-induced degradation is dependent on SCF-βTrCP, whereas IKK β -induced processing of p105 is SCF-βTrCP independent (Cohen et al., 2004). Many studies have suggested that processing of p105 to p50 can occur cotranslationally or posttranslationally (Lin et al., 1998; Lin et al., 2000; Moorthy et al., 2006). A recent report indicates that 20S proteasome endoproteolytically cleaves the full length p105 and degrades the C-terminus of p105, in a ubiquitin-independent manner (Moorthy et al., 2006). When p105 is bound to NF-κB complexes acting like an IκB protein, induced degradation is favored instead of processing (Cohen, 2001).

The non-canonical NF-κB pathway leads to processing of p100, which is signal-dependent and requires IKKα (Senftleben et al., 2001). p100 is phosphorylated at serines 866, 870, and 872, which leads to its subsequent degradation or processing to p52 in a SCF-βTrCP dependent manner (Amir et al., 2004; Liang et al., 2006). p100 can be constitutively processed to p52 in certain cell types in an IKKα dependent manner (Qing and Xiao, 2005).

In contrast to its mammalian counterparts, Relish processing does not depend on proteasomal degradation. Relish is endoproteolytically cleaved by a caspase, producing an N-terminal RHD transcription factor module that translocates to the nucleus to activate immune genes, and a stable C-terminal domain that remains in the cytoplasm (Stöven et al., 2000). Relish cleavage occurs after residue D545, within in a typical caspase target motif. In addition to its role upstream in the IMD pathway, DREDD also appears to function downstream in the pathway and is likely the caspase that cleaves Relish. DREDD and Relish physically interact in cell culture and *Dredd* RNAi prevents

antimicrobial peptide gene expression induced by an activated allele of TAK1 (Zhou et al., 2005). *Dredd* mutants fail to cleave Relish or induce AMP gene expression, and are highly sensitive to Gram-negative bacterial infections (Leulier et al., 2000; Stöven et al., 2003).

The mechanisms involved in the signal-dependent cleavage and activation of Relish are uncertain. Relish is phosphorylated in a signal-dependent manner by the *Drosophila* IKK complex. The C-terminus of Relish is required for both its phosphorylation and cleavage (Stöven et al., 2003), suggesting phosphorylation and cleavage are linked. However, another possibility is raised by the report from Delaney et al. (2006), who showed that TAK1 is not required for Relish cleavage (*in vivo* or in cultured cells), while it was previously reported that the IKK complex, which is activated by TAK1, is necessary for cleavage. This suggested that the IKK complex might control Relish cleavage independently of phosphorylation. In this case, the mechanism(s) by which TAK1-dependent, IKK-mediated phosphorylation regulate Relish remain(s) mysterious, thus this crucial step in IMD pathway is being addressed in this study.

1.6 Thesis Objectives

The long-term objective of the following thesis is to advance our understanding of insect immune response and innate immune mechanisms conserved from insects to humans, using *Drosophila* as a model system. The studies presented here were designed to elucidate the molecular mechanism of activation of *Drosophila* NF-κB precursor Relish and its regulation by the *Drosophila* IκB kinase complex. The findings of this research could contribute to better understanding of vertebrate and invertebrate innate immunity as well as to prevention and therapeutics of infectious diseases and immune system disorders.

Figure 1.1

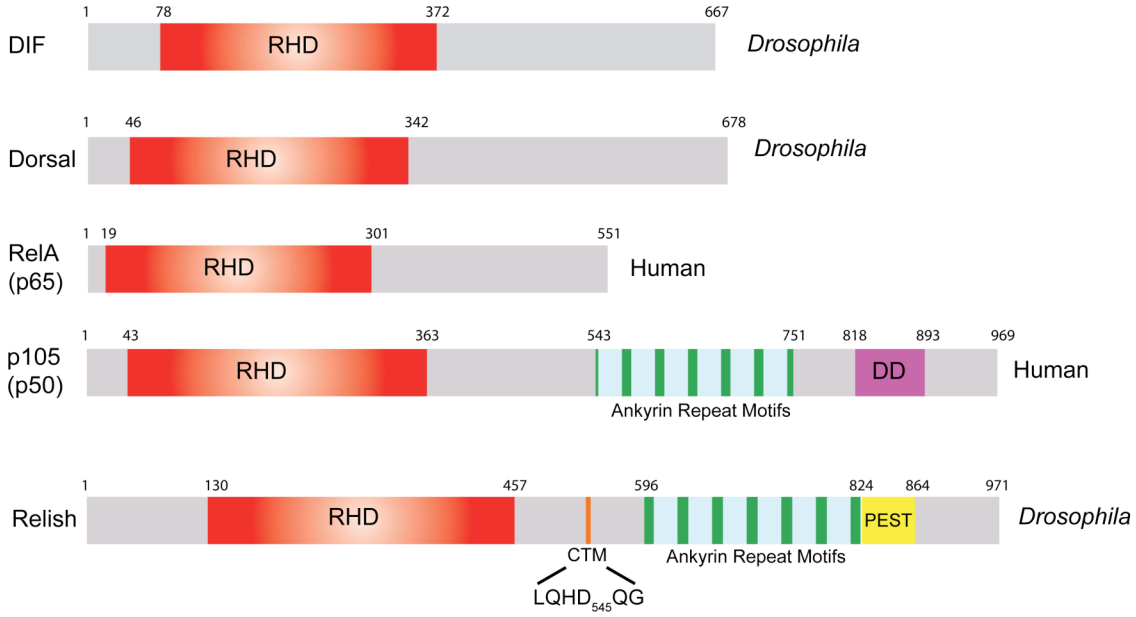

DIF
1
78
372
667
RHD
Drosophila
Dorsal
1
46
342
678
RHD
Drosophila
RelA
(p65)
1
19
301
551
RHD
Human
p105
(p50)
1
43
363
543
751
818
893
969
RHD
DD
Human
Ankyrin Repeat Motifs
Relish
1
130
457
596
824
864
971
RHD
PEST
Drosophila
CTM
Ankyrin Repeat Motifs
LQHD545QG

Figure 1. 1 Members of the NF-κB family in mammals and *Drosophila*

Drosophila encodes three NF-κB family members. DIF and Dorsal are similar to mammalian RelA (p65), and Relish is similar to mammalian NF-κB precursor p50/p105. All NF-κB family members share an N-terminal Rel-homology domain (RHD) that mediates DNA binding, dimerization and interaction with IκB proteins. Contrary to mammalian NF-κB precursors that require proteasome degradation, Relish processing depends on caspase cleavage. Cleavage occurs after residue D545, within in a caspase target motif (CTM).

Figure 1.2

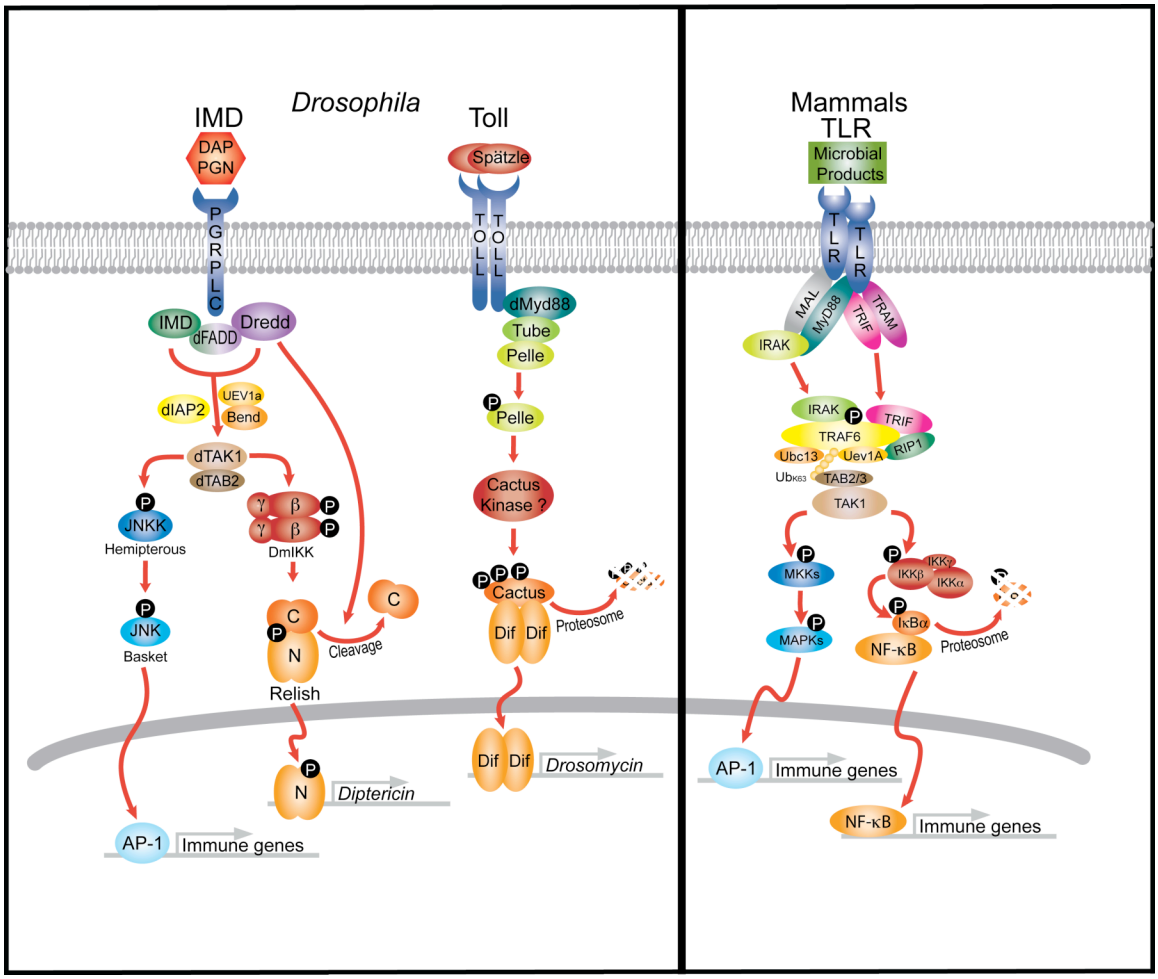

Drosophila
IMD
DAP
PGN
PGRPLC
IMD
dFADD
Dredd
dIAP2
UEV1a
Bend
dTAK1
dTAB2
JNKK
Hemipterous
JNK
Basket
DmIKK
C
N
Cleavage
C
Relish
N
Diptericin
AP-1
Immune genes
Toll
Spätzle
TOLL
TOLL
dMyd88
Tube
Pelle
Pelle
Cactus
Kinase ?
Cactus
Dif Dif
Proteosome
Dif Dif
Drosomycin
Mammals
TLR
Microbial
Products
TLR
TLR
MAL
MyD88
TRIF
TRAM
IRAK
IRAK
TRIF
TRAF6
Ubc13
Uev1A
RIP1
Ub
TAB2/3
TAK1
MKKs
MAPKs
IKKγ
IKKβ
IKKα
IκBα
NF-κB
Proteosome
AP-1
Immune genes
NF-κB
Immune genes

Figure 1. 2 Innate immune signaling in *Drosophila* and mammals

Recognition of Gram-negative PGN by the PGRP-LC receptor leads to signaling of the IMD pathway (left). IMD signaling shows homology to mammalian MyD88 independent, Trif dependent TLR signaling (right). After activation of the receptor and adaptors the JNK and NF-κB/Relish branches of the IMD pathway are activated in a TAK1 dependent manner. Activation of these two branches leads to the rapid induction of various immune and AMP genes. The presence of Gram-positive bacteria and fungi lead to the proteolytic cleavage of pro- Spätzle. Mature Spätzle then acts as the *Drosophila* Toll ligand (center). Unlike IMD, Toll signaling shows homology to mammalian MyD88 dependent signaling. Activation of the Toll receptor leads to the recruitment of the dMyd88/Tube/Pelle adaptor complex. An unidentified Cactus kinase then phosphorylates the IκB protein Cactus, which is proteolytically degraded. Degradation of Cactus then allows the NF-κB protein DIF (or Dorsal) to translocate into the nucleus activating various AMPs.

CHAPTER II:

The Death Domain of Relish is Essential for its Interaction with IKKβ

Abstract:

The *Drosophila* Rel/NF-κB transcription factor Relish is required for IMD pathway that responds to Gram-negative bacterial infections. Relish is a bipartite precursor protein consisting of an N-terminal Rel homology domain and a C-terminal IκB-like domain. Gram-negative bacterial peptidoglycan (PGN) stimulates IMD pathway that leads to activation of *Drosophila* IκB Kinase complex followed by phosphorylation and endoproteolytic cleavage of Relish. Upon cleavage, the N-terminal Rel homology domain translocates into the nucleus and activates antimicrobial peptide genes whereas the C-terminal IκB-like domain remains intact in the cytoplasm. It has been shown that DmIKKβ can phosphorylate Relish *in vitro* and the IKK complex is required for signal-dependent cleavage of Relish and production of antimicrobial peptides. Here we present that C-terminal 107 amino acids of Relish is required for its phosphorylation *in vitro* and its cleavage in S2 cells. However, this region is not a target of phosphorylation but instead is an interaction domain with the DmIKKβ. Furthermore, sequence-structure homology recognition suggests that C-terminus of Relish contains a putative death domain. Small deletions in this region or mutating a highly conserved tryptophan residue to alanine (W914A) can block interaction of Relish with IKKβ suggesting an essential role for the death domain of Relish.

Introduction:

Humoral immune responses in *Drosophila* are controlled by two immune signaling pathways. IMD pathway is triggered by Gram-negative bacterial infections, whereas Toll pathway responds to Gram-positive and fungal infections (Cherry and Silverman, 2006; Kaneko et al., 2005; Lemaitre and Hoffmann, 2007; Leulier and Lemaitre, 2008). Both pathways result in induction of antimicrobial peptide genes, which are transcriptionally regulated by Rel/NF-kappa B family members DIF and Dorsal in Toll pathway and Relish in IMD pathway (Minakhina and Steward, 2006).

Relish is a precursor protein composed of an N-terminal Rel homology domain and a C-terminal IκB-like domain, similar to the mammalian NF-κB precursors p100 and p105. Activation of the IMD pathway results in phosphorylation and caspase-dependent endoproteolytic cleavage of Relish. Upon cleavage, N-terminal NF-κB transcription module translocates to the nucleus to activate antimicrobial peptide genes, such as *Diptericin* and the C-terminal IκB-like domain remains intact in the cytoplasm (Hedengren et al., 1999; Stöven et al., 2000).

Phosphorylation and endoproteolytic cleavage of Relish requires the *Drosophila* IKK complex. The IKK complex is composed of a catalytic kinase subunit encoded by *ird5* (IKKβ) and a regulatory subunit encoded by *kenny* (IKKγ) (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000). The activated IKK complex or the recombinant IKKβ can directly phosphorylate Relish *in vitro* (Silverman et al., 2000). Furthermore, *ird5* or *kenny* mutant larvae fail to cleave Relish.

Here we present that the C-terminal 107 residues of Relish are required for both

its phosphorylation *in vitro* and cleavage in cells. However, this region is not the target of DmIKKβ-mediated phosphorylation. Instead, C-terminus of Relish is the interaction site with IKKβ. Furthermore, Relish C-terminus shows homology to other death domain proteins, suggesting that Relish and IKKβ interaction is mediated by Relish death domain.

Death domains (DD) are 80-100 residue long motifs mediating protein interactions in various signaling pathways that regulate apoptosis, development and immunity (Cleveland and Ihle, 1995; Feinstein et al., 1995; Weber and Vincenz, 2001). Death domain is first identified as a cytoplasmic domain conserved between Fas and TNF-R1 and is necessary for transduction of the apoptotic signal (Nagata and Golstein, 1995; Suda et al., 1993). The group of death domain proteins is a member of death domain fold superfamily consisting of four subfamilies: the death domain (DD), the death effector domain (DED), the caspase recruitment domain (CARD), and the PYRIN domain (PYD) proteins (Park et al., 2007a). Interaction of death domain containing proteins regulates the activity of several signaling proteins, such as receptors, adaptors, caspases and transcription factors. Recent studies demonstrate that death domains are involved in not only homotypic, but also heterotypic interactions among different sub-families, and even non-death domain fold interactions. Sequence similarity among death domain proteins is so low that it is not considered statistically significant by conventional sequence comparison methods. However all members of the DD superfamily share a conserved structural fold. A typical death domain fold consists of six helices arranged in an anti-parallel manner forming a tightly packed hydrophobic core. The surface features of the death domains are different due to low sequence homology, which may be

responsible for specificity of the protein-protein interaction interfaces.

Using a multiple alignment program for aminoacid sequences (MAFFT version 6) with a multiple alignment editor (Jalview) and a sequence-structure homology recognition program (FUGUE) , we have identified a putative death domain in the C-terminus of Relish and modeled its structure. Small internal deletions in this region or mutating a highly conserved tryptophan residue to alanine (W914A) can block interaction between Relish and IKKβ validating the importance of Relish death domain.

Results:

Relish is phosphorylated in a signal-dependent manner

It has been demonstrated previously that the activated IKK complex and the recombinant IKKβ can directly phosphorylate Relish *in vitro* (Silverman et al., 2000). In order to analyze the relationship between Relish and IKK complex, we first wanted to show if Relish is indeed phosphorylated in a signal-dependent manner in S2* cells.

The caspase inhibitor zVAD-FMK blocks Relish processing in *Drosophila* cell culture resulting in an increase in the apparent molecular weight of REL-110 upon stimulation with PGN. This modification could be reversed by phosphatase treatment (Figure 2-1). Thus, Relish is phosphorylated in a signal-dependent manner prior to or during its proteolytic cleavage. The phosphorylation is likely to be mediated by the IKK complex, which can directly phosphorylate Relish *in vitro.*

The C-terminus of Relish is required for *in vitro* phosphorylation and signal-dependent cleavage in cells.

Typically, signals that induce NF-κB activity cause the phosphorylation of IκB proteins, their dissociation and subsequent degradation, allowing NF-κB proteins to enter the nucleus and induce gene expression. Similarly C-terminal IκB-like domain of Relish could also be important for its phosphorylation and cleavage. In order to test that, wild type and C-terminal truncation mutants (ΔC824 and ΔC865) of Relish were generated by *in vitro* translation and used as a substrate in kinase assay with recombinant IKKβ. Wild

type Relish was robustly phosphorylated, whereas C-terminal truncations strongly inhibited IKKβ-mediated phosphorylation *in vitro* (Figure 2-2A). In stable cell lines, signal-induced cleavage of these truncation mutants was also greatly reduced suggesting that C-terminus of Relish is phosphorylated to control its activation by caspase-dependent cleavage (Figure 2-2B).

The C-terminus of Relish is not a major target of IKK-mediated phosphorylation.

Surprisingly, when all 10 serines and threonines in this 107 amino acid C-terminal region were changed to alanine, Relish was still phosphorylated *in vitro* (Figure 2-3A) and was cleaved in response to immune stimulation in S2* cells normally (Figure 2-3B). Thus, the C-terminus of Relish is not a major target of IKK-mediated phosphorylation and phosphorylation of this region is not required for signal-induced cleavage. One possible explanation to this result is that the C-terminus of Relish is the interaction site with IKKβ.

The C-terminus of Relish is required for interaction with IKKβ

In order to test if Relish and IKKβ interact via the C-terminus of Relish, co-immunoprecipitation of IKK complex with Relish wild type and C-terminal truncation mutants were analyzed. FLAG tagged Relish was immunoprecipitated with FLAG antibody and immunoblotted with IKKγ antibody. IKK complex co-immunoprecipitates with wild type Relish but not with C-terminal mutants showing that Relish C-terminus is required for interaction with the IKK complex (Figure 2-4A). To analyze direct

interaction of Relish with IKKβ, we have used yeast two-hybrid analysis (James et al., 1996). Relish and IKKβ were co-transformed in yeast and interaction was assayed with growth on adenine-free medium. IKKβ can interact with wild type Relish but not with C-terminal truncations, demonstrating that the interaction between Relish and IKKβ is mediated by Relish C-terminus. Mutation of ten phosphoacceptor residues in this region does not affect interaction (Figure 2-4B).

Sequence and structure analysis of Relish C-terminus identifies a death domain

It has been recently published that the *Aedes aegypti* Relish contains a death domain in its C-terminus similar to other known death domain proteins. However, the C-terminus of *Drosophila* Relish showed a low sequence homology to death domain proteins in that alignment (Shin et al., 2002). A growing literature on death domain proteins indicates that the sequence similarity across the death domain superfamily is low, and it does not always correlate with structural similarity (Cleveland and Ihle, 1995). Therefore we addressed the possibility of Relish having a functional death domain by sequence and structural analysis.

In order to test the presence of a death domain in Relish, we first examined conservation of the aminoacid sequence of Relish C-terminus. First, the C-terminal region of Relish was aligned among 12 *Drosophila* species whose genomes are sequenced. The protein sequence of *Drosophila melanogaster* Relish is almost identical to *Drosophila simulans* and *Drosophila sechellia*, which are in melanogaster subgroup and it also shows very high homology to other species (Figure 2-5A). Then, *Drosophila* Relish is aligned to C-terminal sequences of *Aedes* and *Anopheles* Relish as well as other

death domain proteins with known structures using MAFFT-multiple sequence alignment program (Figure 2-5A). The overall sequence homology of Relish to other proteins is low with only one identical tryptophan residue at position 914. C-terminal sequence of Relish is most homologous to *Aedes aegypti,* with 20 percent identity and 34 percent similarity. However, when the C-terminus of Relish is modeled by a sequence-structure homology recognition program (FUGUE) using the death domains of the human NF-κB p100 and p105 as templates, it folds into a typical death domain structure highly similar to other death domain proteins with six antiparallel helices folded to form a compact core structure (Figure 2-6). The six α-helices of Relish are shown at the top of the alignment blocks (Figure 2-5).

Death domain structure of Relish is required for interaction with IKKβ

In order to test if Relish death domain is required for its interaction with IKKβ, deletions of twenty aminoacids each were generated in this region starting form aminoacid 824. The first two deletions (Δ824-865 and Δ886-906), which do not cover the death domain, can interact with IKKβ normally, whereas all other deletions spanning the predicted death domain inhibit interaction between Relish and IKKβ (Figure 2-5C). In order to rule out that the lack of interaction is due to gross structural changes, point mutations of the highly conserved tryptophan residue at position 914 were generated. The tryptophan 914, which has a large aromatic sidechain, is located at the beginning of the second helix in the core structure. Trp914 residue is mutated to alanine to locally unfold the death domain structure and back mutated to phenylalanine, which also has an aromatic side chain similar to tryptophan (Figure 2-7A). Those mutants were tested for interaction with

IKKβ using yeast two-hybrid system. W914A mutation inhibits interaction with IKKβ, and the yeast cells cannot grow on adenine-free medium. On the other hand, mutating W914 back to a structurally similar, aromatic residue phenylalanine, restores the interaction, demonstrating that the death domain structure is important for Relish IKKβ interaction (Figure 2-7B).

Identifying critical residues in the Relish death domain

In order to identify residues potentially involved in protein-protein interactions between Relish and IKKβ, amino acids predicted to be on the surface of the death domain were examined. Based on sequence conservation and structure models, H905, D908, D911, E934 and D935 with predicted electronegative surface and K924 and R954 with predicted electropositive surface were identified as residues that might be important for a potential binding interface. L895, F952 and Y964 are other important residues, which may affect the structure of hydrophobic core.

Discussion

Gram-negative bacterial infections stimulate IMD pathway, which results in production antimicrobial peptides against invading microorganisms. Induction of antimicrobial peptides in IMD pathway is regulated by the Rel/NF-κB transcription factor Relish, which is a bipartite precursor protein consisting of an N-terminal Rel homology domain and a C-terminal IκB-like domain. Upon stimulation of the IMD pathway, the signaling cascade results in activation of *Drosophila* IKK complex followed by phosphorylation and endoproteolytic cleavage of Relish releasing the N-terminal transcription module, which in turn translocates into the nucleus and activates antimicrobial peptide genes.

It has been shown that DmIKKβ can phosphorylate Relish *in vitro* and the IKK complex is required for signal-dependent cleavage of Relish and production of antimicrobial peptides. However, details of the relationship between IKK complex and Relish have not been determined. Here we demonstrate that Relish is phosphorylated in a signal-dependent manner *in vivo* and the C-terminal 107 amino acids of Relish are required for its phosphorylation *in vitro* and its cleavage in S2 cells. This result first suggested that phosphorylation and cleavage are related and phosphorylation of Relish C-terminus controls signal-dependent cleavage of Relish. However, when we mutated all the phosphoacceptor sites in this region, 6 serines and 4 threonines, Relish was still phosphorylated *in vitro* and cleaved in S2* cells normally showing that Relish C-terminus is not a target of phosphorylation. Using co-immunoprecipitaion and yeast two-

hybrid analyses, we demonstrated that C-terminus of Relish is an interaction domain with IKKβ.

Furthermore, using sequence-structure homology recognition program (FUGUE) we have modeled a putative death domain in Relish C-terminus. The death domain (DD) modules are important protein–protein interaction domains found in various signaling proteins including receptor, adaptor, effector, and inhibitor proteins involved in various cellular processes such as apoptosis, inflammation and development. These interaction domains have low sequence homology but share a common three-dimensional fold, named as the death domain fold and characterized by a highly compact structure with six antiparallel α-helices. The helices enclose a tightly packed hydrophobic core. Predicted model for the structure of Relish C-terminus is highly similar to other proteins with known death domains suggesting that interaction of Relish with IKKβ is mediated by its death domain fold.

Mutations in the Relish death domain validate the importance of that structure. Small deletions in the region or alanine substitution of a conserved tryptophan residue (W914A) block interaction of Relish with IKKβ suggesting an essential role for the putative death domain of Relish. However, deletions may be affecting the overall structure of Relish. Also, due to its location in the core of the death fold and its large aromatic side chain, mutations of W914 is expected to cause local unfolding of the death domain rather than abolishing interaction interface between Relish and IKKβ. Partial restoration of the interaction with an aromatic residue substitution (W914F) supports that possibility.

In order to identify the interaction interface between Relish and IKKβ, amino acids predicted to be on the surface were examined and potentially important residues were identified (H905, D908, D911, E934, D935, K924 and R954). These residues need to be further analyzed by mutations and interaction assays to verify their role in interaction between Relish and IKKβ.

Recent reports demonstrate that death domains are involved in not only homotypic-, but also heterotypic and non-death domain interactions, in various signaling molecules such as receptors, adaptors and transcription factors including NF-κB proteins. For example, the death domain of NF-κB1 p105 in the C-terminus is essential for its proteolysis. Mutations in the death domain of NF-κB1 inhibit its interaction with IKK and its signal-induced phosphorylation and proteolysis (Beinke et al., 2002). Despite low sequence similarity among death domain proteins, the death domain fold with six helices is evolutionarily conserved. Our data demonstrate that Relish C-terminus contains a death domain, which is essential for interaction with IKKβ. Further studies are needed to determine the interaction interface between Relish and IKKβ.

Materials and Methods:

Plasmids

Deletions and point mutations were generated by PCR-based site directed mutagenesis. FLAG-tagged WT, D824, D865 and 6SA4TA mutant Relish were cloned into pCITE-2a(+) with T7 promoter for *in vitro* translation and pPacPL with actin promoter for cellular expression using standard methods. For yeast two-hybrid analysis, IKKβ was cloned into pGBDU Gal4 binding domain plasmid with URA marker and various Relish constructs were cloned into pGAD-C1 Gal4 activation domain plasmid with LEU marker (James et al., 1996).

Cell culture

Drosophila S2*cells were grown in Schneiders Media (Gibco) with 10% Fetal Bovine Serum, 1% Glut-MAX (Gibco) and 0.2% Penicillin-Streptomycin (Gibco) at 27°C. Cells were treated with 1μM 20-hydroxyecdysone for 24 hours before PGN stimulation (Invivogen).

DNA transfections and stable cell lines

S2* cells were plated at the density of $1x10^6$ cells/ml. The cells were transfected with 1.5 mg/ml DNA or RNA using calcium phosphate transfection method. 24 h later, the cells were split to $1x10^6$ cells/ml and treated with 20-hydroxyecdysone at 1μM for 24 h. Cells were then stimulated with peptidoglycan (Invivogen) for 10 minutes for Relish cleavage.

For stable cell lines, pPacPL constructs with actin promoter were transfected into Schneider S2* cells in conjunction with pHS-neo, 50:1; stable transfectants were then selected with G418 (800 μg/mL)**.**

Protein analysis

For Western Blot analysis, cells or were lysed in lysis buffer (20mM Tris pH 7.6, 150 mM NaCl, 25mM β-glycerophosphate, 2mM EDTA, 10% glycerol, 1% Triton X-100, 1mM DTT, 1mM $NaVO_4$, 1X Protease inhibitor cocktail (sigma), 100mM Okadaic acid) total protein extracts were separated by SDS-PAGE and transferred to PVDF membrane. Antibodies used for immunoprecipitation or immunoblotting were as follows: anti-FLAG (sigma).

Kinase assay

Versions of Relish were translated *in vitro* in reticulocyte lysates (Promega) and then immunoprecipitated by using anti-FLAG agarose (Sigma). 1/3 of these immunoprecipitates were used in the control Western blot, the rest in an *in vitro* kinase reaction with recombinant *Drosophila* IKKβ (Silverman, 2000) and γ-^{32}P-ATP. Kinase reactions were performed in kinase buffer (20 mM Hepes at pH 7.6, 20 mM beta - Glycerophosphate, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, 0.1 mM NaVO4, 200 μM ATP, and 5 μCi γ-^{32}P-ATP).

Yeast two-hybrid assay

PJ49a strain was used for co-transformations of Relish and IKKβ. A 200 ml liquid culture was grown overnight at 30°C. Yeast was pelleted and washed with distilled water. 50µl of cell pellet was used for each transformation. 240 µl of 50% w/v PEG 3500, 36 µl of 1.0 M LiAc, 50 µl of boiled SS-Carrier DNA (2 mg/ml) and 34 µl Plasmid DNA (0.1 to 1 µg) plus water were added on top of the pellet in a total volume of 360 µl and incubated at 42°C for 60 min. Cells were plated on Ura-/Leu-plates and contransformants were tested for interaction by growing on Ade- plates

Sequence and structure analysis

The protein sequences were obtained from FlyBase or NCBI were aligned using multiple alignment program (MAFFT) and multiple alignment editor (Jalview). Protein structure data of proteins with known death domains were obtained from protein data bank (PDB). Death domain structure of Relish was modeled by FUGUE- a sequence-structure homology recognition program, using the death domains of the human NF-κB p100 and p105 subunits (pdb 2d96 and 2dbf) as templates. Open source molecular graphics package PyMOL was used to create structural representations.

Figure 2-1

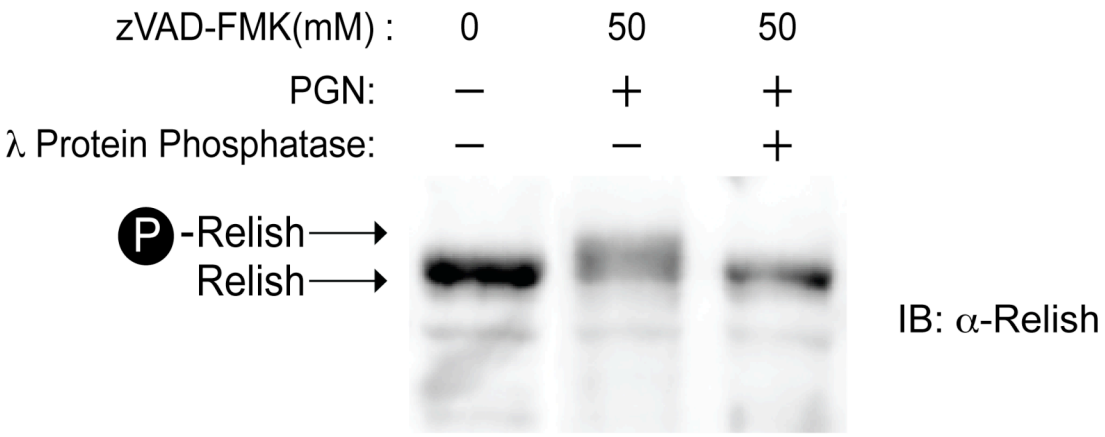

zVAD-FMK(mM) :
0
50
50
PGN:
−
+
+
λ Protein Phosphatase:
−
−
+
P-Relish
Relish
IB: α-Relish

Figure 2. 1 Relish is phosphorylated upon PGN stimulation

The Signal-dependent modification of Relish is due to phosphorylation. The caspase inhibitor zVAD-FMK blocks peptidoglycan (PGN)-stimulated Relish cleavage in S2* cells resulting in accumulation of a slower migrating form of Relish. Modification of endogenous Relish from caspase inhibitor treated cell extract is reversed by λ protein phosphatase.

Figure 2-2

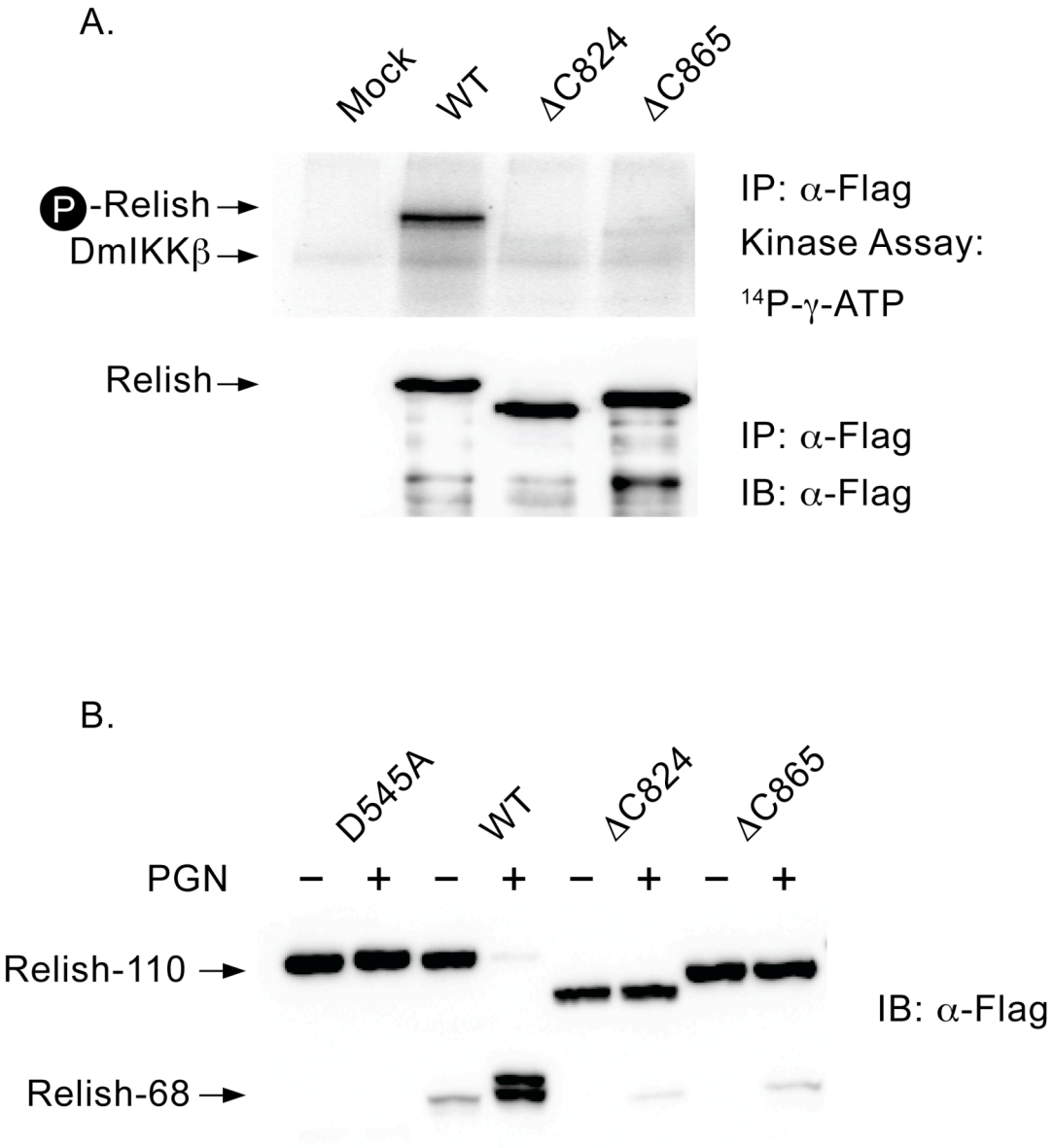

A.
Mock
WT
ΔC824
ΔC865
P-Relish
DmIKKβ
IP: α-Flag
Kinase Assay:
14P-γ-ATP
Relish
IP: α-Flag
IB: α-Flag
B.
D545A
WT
ΔC824
ΔC865
PGN
– + – + – + – +
Relish-110
Relish-68
IB: α-Flag

Figure 2. 2 The C-terminus of Relish is required for its phosphorylation and cleavage

A. C-terminal truncations inhibit IKK-mediated *in vitro* phosphorylation of Relish. Wild type and C-terminally truncated mutant versions of Relish were translated *in vitro*, immunoprecipitated, and used in kinase reaction with recombinant DmIKKβ. Translation and immunoprecipitation were confirmed by western blot. **B.** C-terminus of Relish is required for PGN-induced cleavage of Relish in stable S2* cell lines. Cleavage of wild type and mutant Relish proteins, in stably transfected *Drosophila* S2* cells, following peptidoglycan stimulation was analyzed by immunoblotting.

Figure 2-3

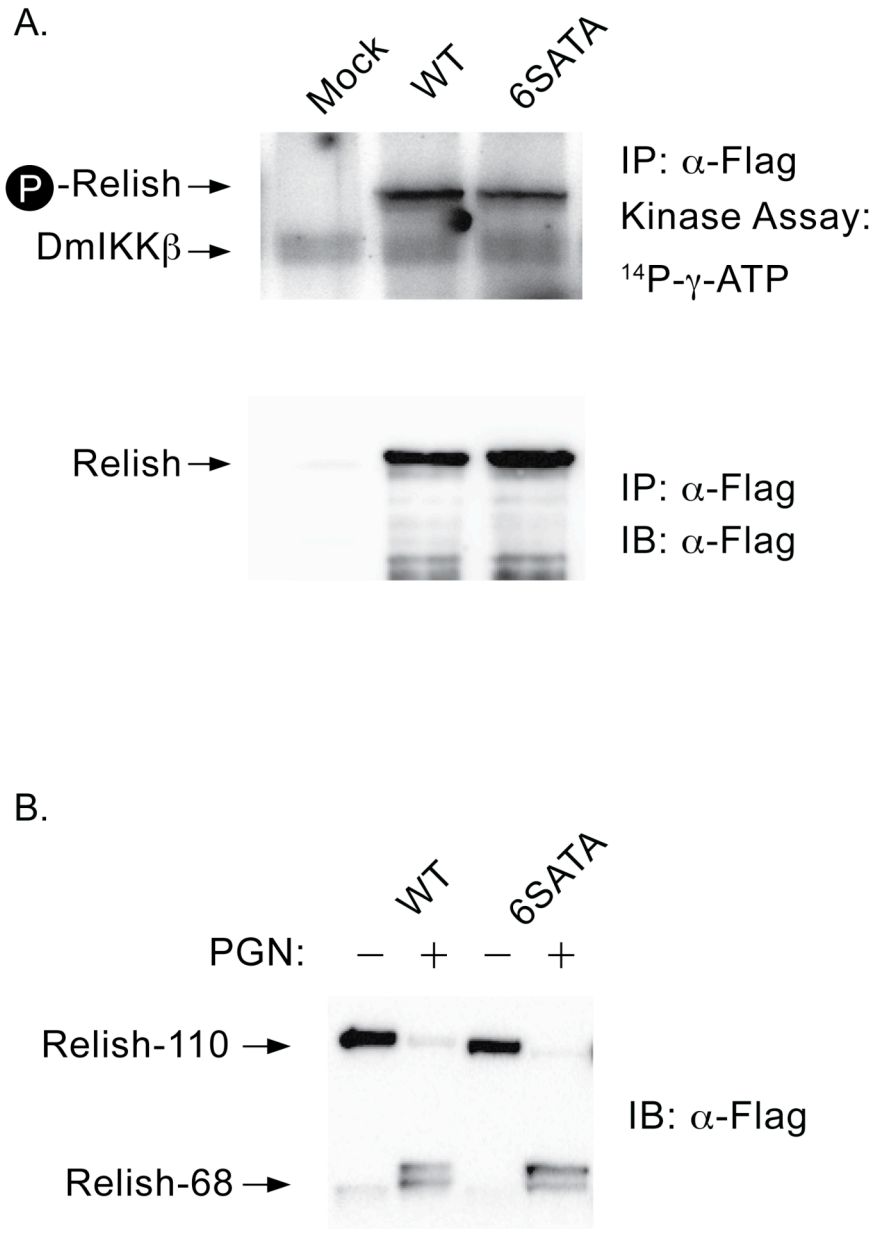
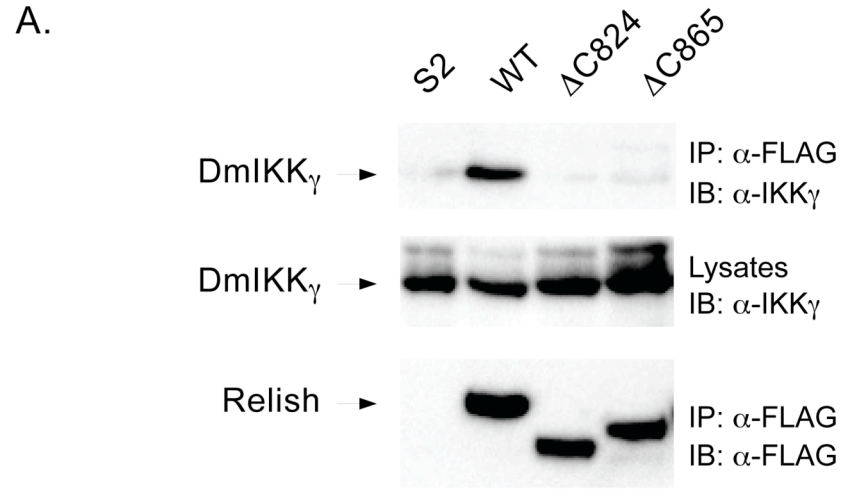

A.
Mock
WT
6SATA
P-Relish
DmIKKβ
IP: α-Flag
Kinase Assay:
14P-γ-ATP
Relish
IP: α-Flag
IB: α-Flag
B.
WT
6SATA
PGN: – + – +
Relish-110
Relish-68
IB: α-Flag

Figure 2. 3 C-terminal phosphoacceptor residues are not required for IKK-mediated phosphorylation and signal-induced cleavage of Relish

A. 6SA4TA mutant Relish is phosphorylated by IKK *in vitro*. Wild type and mutant (with ten phosphoacceptor residues in C-terminal 107 amino acids region changed to alanine - S871A, S872A, T875A, S897A, S907A, T921A, S946A, S950A, T951A, T971A (6SA4TA)) versions of Relish were translated *in vitro*, immunoprecipitated with FLAG-antibody, and used as a substrate in kinase reaction with recombinant *Drosophila* IKKβ. 1/3 of immunoprecipitated translation reaction was immunoblotted with FLAG antibody to confirm translation and immunoprecipitation (lower panel) **B**. C-terminal phosphoacceptor sites are not required for PGN-induced cleavage in S2* cells. Cleavage of wild type and mutant Relish proteins, in stably transfected *Drosophila* S2* cells, was analyzed by immunoblotting using FLAG antibody before and after PGN-stimulation.

Figure 2-4


A.
S2
WT
ΔC824
ΔC865
DmIKKγ
IP: α-FLAG
IB: α-IKKγ
DmIKKγ
Lysates
IB: α-IKKγ
Relish
IP: α-FLAG
IB: α-FLAG

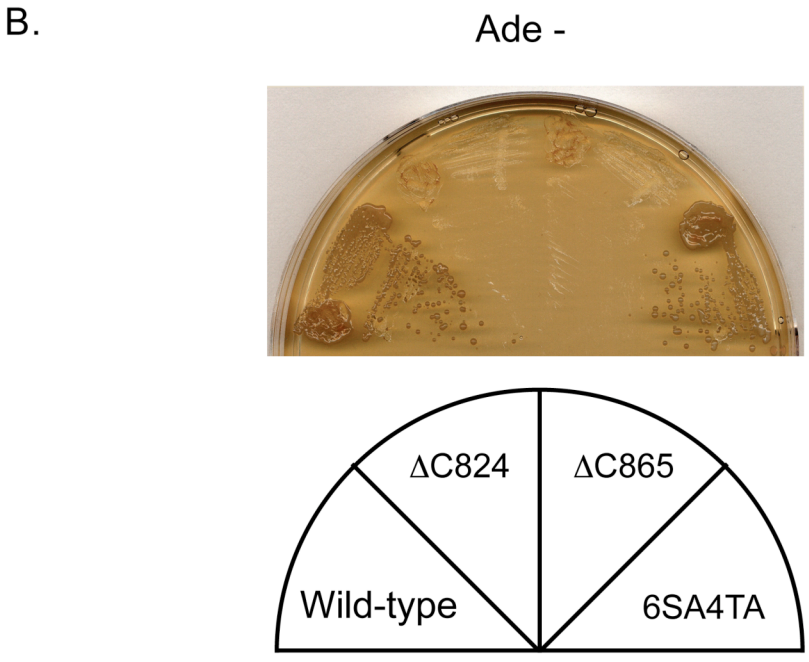

B.
Ade -
ΔC824
ΔC865
Wild-type
6SA4TA

Figure 2. 4 Relish C-terminus is required for interaction with IKK

A. Truncation mutants in the C-terminus inhibit association of IKKγ with Relish. Interaction between endogenous IKKγ and Relish was analyzed by coimmunoprecipitation followed by western blot analysis using stable cell lines that express wild type and mutant versions of Relish. **B.** C-terminus of Relish is required for direct interaction with IKKβ. Interaction between DmIKKβ and Relish was analyzed by yeast two-hybrid system using DmIKKβ as a bait, wild type and mutant versions of Relish as preys. They were co-expressed in a yeast strain that has ADE2 reporter. Growth on adenine-deficient medium is indicated.

Figure 2-5

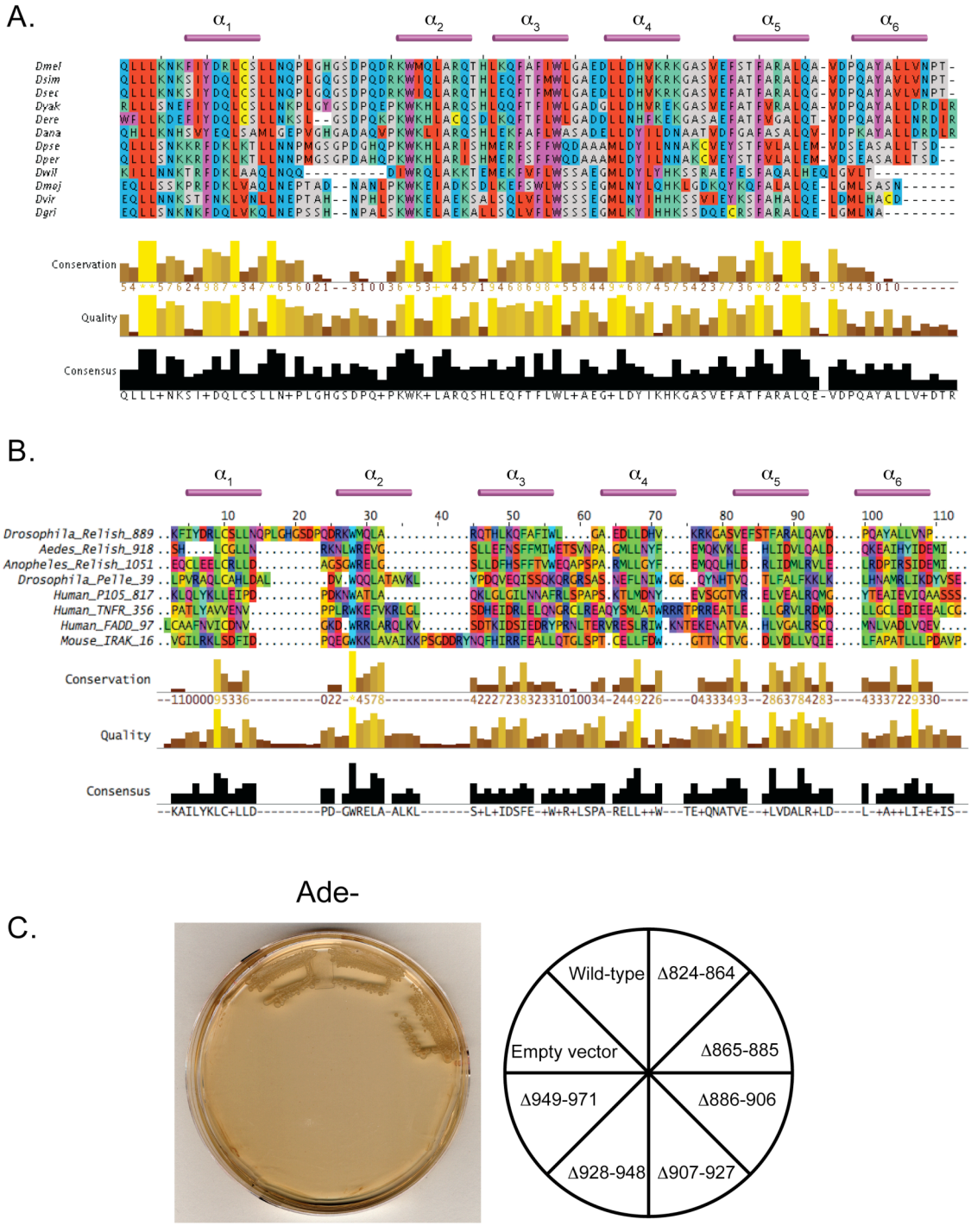

A.
α1
α2
α3
α4
α5
α6
Dmel
Dsim
Dsec
Dyak
Dere
Dana
Dpse
Dper
Dwil
Dmoj
Dvir
Dgri
Conservation
Quality
Consensus
B.
Drosophila_Relish_889
Aedes_Relish_918
Anopheles_Relish_1051
Drosophila_Pelle_39
Human_P105_817
Human_TNFR_356
Human_FADD_97
Mouse_IRAK_16
Conservation
Quality
Consensus
C.
Ade-
Wild-type
Δ824-864
Empty vector
Δ865-885
Δ949-971
Δ886-906
Δ928-948
Δ907-927

Figure 2. 5 C-terminus of Relish contains a putative death domain

C-terminus of Relish is highly conserved among other *Drosophila* species. Relish sequences from twelve *Drosophila* species: *Drosophila melanogaster, Drosophila simulans, Drosophila sechellia, Drosophila yakuba, Drosophila erecta, Drosophila ananassae, Drosophila pseudoobscura, Drosophila persimilis, Drosophila willistoni, Drosophila mojavensis, Drosophila virilis and Drosophila grimshawi* were obtained from FlyBase and aligned using multiple alignment program (MAFFT) and multiple alignment editor (Jalview). **B.** C-terminus of Relish shows some homology to other death domain proteins. The C-terminal aminoacid sequence of *Drosophila melanogaster* Relish was compared to, *Aedes aegypti* (AF498105) and *Anopheles gambiae* (AY353563) Relish C-terminal sequences and to other proteins with known death domain structures: *Drosophila* Pelle (L08476), human NF-κB p105 (M55643), human TNFR (X55313), human FADD (U24231) and mouse IRAK (AF445803), using multiple alignment program (MAFFT) and multiple alignment editor (Jalview). **C.** Interaction of Relish with IKKβ requires the death domain. Twenty aminoacid deletions spanning the C-terminus of Relish (Δ824-864, Δ865-885-, Δ886-906, Δ907-927, Δ928-948, Δ949-971) were made and tested for interaction with DmIKKβ using yeast two-hybrid system.

Figure 2-6

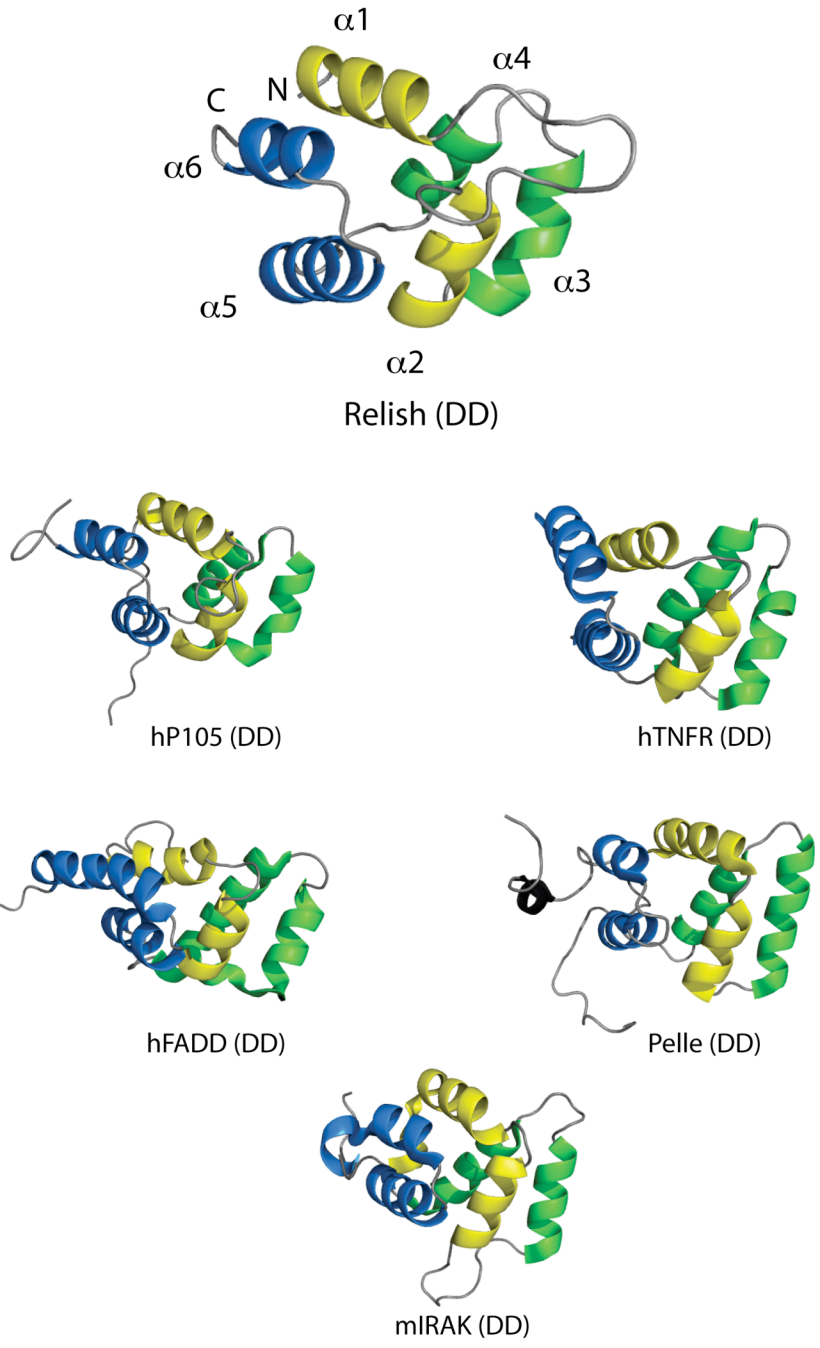
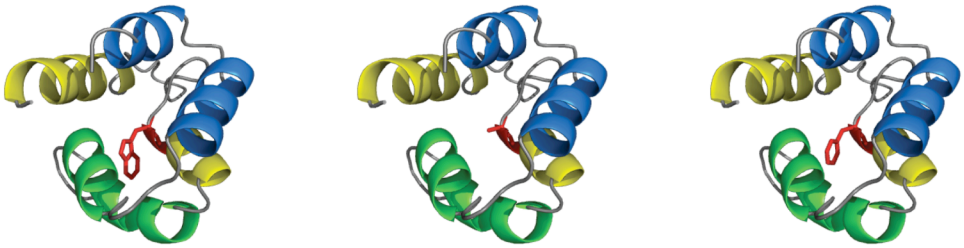

α1
α4
C
N
α6
α3
α5
α2
Relish (DD)
hP105 (DD)
hTNFR (DD)
hFADD (DD)
Pelle (DD)
mIRAK (DD)

Figure 2. 6 Relish death domain model

Predicted model for Relish death domain resembles other death domain protein structures. Relish C-terminus is modeled by FUGUE- a sequence-structure homology recognition program, using the death domains of the human NF-κB p100 and p105 subunits (pdb 2d96 and 2dbf) as templates. The ribbon diagrams illustrate the secondary structure elements (yellow: helices 1 and 2, green: helices 3 and 4, blue: helices 5 and 6). Death domain structures of human NF-κB p105 (pdb 2dbf), human TNFR1 (pbd ich), human FADD (pbd 1e3y), *Drosophila* Pelle (pbd 1ygo), mouse IRAK (pbd 2a9i) are shown for comparison.

Figure 2-7

A.



W914 W914A W914F

B.

Ade-

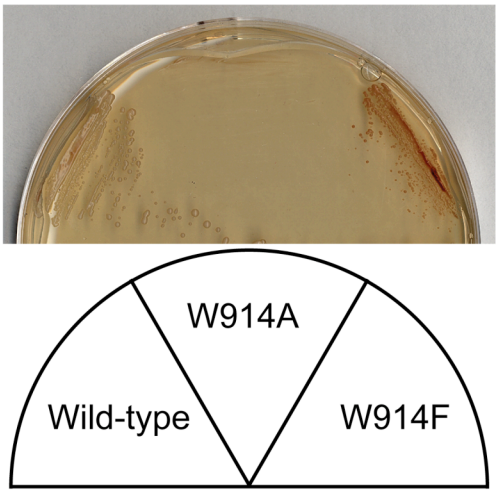
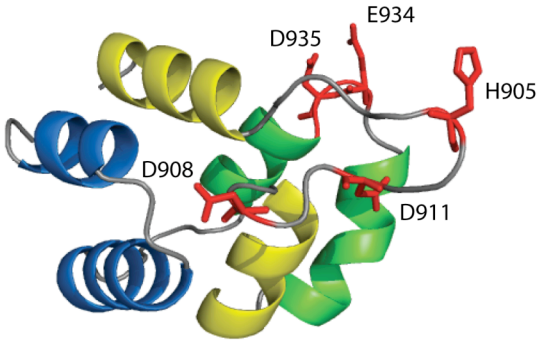


Figure 2. 7 The W914A mutation in the death domain of Relish inhibits interaction with IKKβ

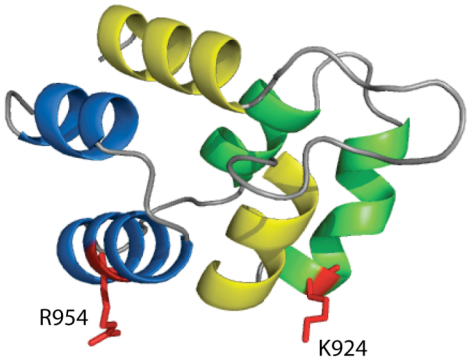
A. Ribbon diagram of death domain of Relish showing the aminoacid side chains of the highly conserved tryptophan residue and its substitutions to alanine and phenylalanine (W914A and W914F) found at the beginning of helix 2. **B.** Interaction of Relish with IKKβ requires W914. W914A and W914F mutations were generated by site directed mutagenesis and tested for interaction with in the DmIKKβ using yeast two-hybrid system. Drastic mutation of tryptophan to alanine inhibit interaction with IKKβ, while back mutation to phenylalanine, a similar aromatic residue partially restored interaction.

Figure 2-8

A.



B.



C.

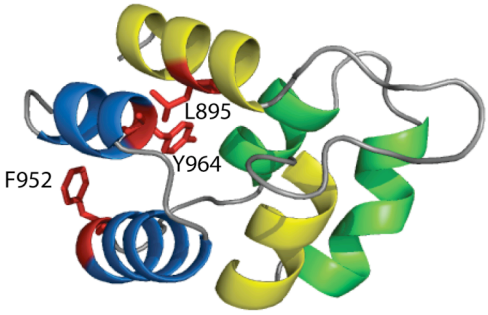


Figure 2. 8 Prediction of critical residues in Relish death domain

Critical residues predicted based on the modeled structure of Relish death domain, potential intra-molecular contacts and sequence conservation to other death domain proteins are presented with their aminoacid sidechains (red) **A.** Residues with predicted electronegative surface that might be important for potential binding interface are H905, D908, D911, E934 and D935. **B.** Residues with predicted electropositive surface that might be important for potential binding interface are K924 and R954. **C.** Residues that might be important for destabilization of hydrophobic core are L895, F952 and Y964 .

CHAPTER III:

The *Drosophila* IκB Kinase Controls Transcription of Antimicrobial Peptide Genes by Direct Phosphorylation of Relish

Abstract:

Relish, a *Drosophila* NF-κB transcription factor, is an essential regulator of antimicrobial peptide gene induction following Gram-negative bacterial infection. Relish is a bi-partite NF-κB precursor protein, with an N-terminal Rel homology domain and a C-terminal IκB-like domain, similar to mammalian p100 and p105. In response to bacterial infections, Relish is endoproteolytically cleaved and the NF-κB module translocates to the nucleus. Signal-dependent activation of Relish, including cleavage and nuclear translocation, requires the *Drosophila* IκB kinase complex (IKK) and DREDD, the *Drosophila* caspase-8 like protease. In this report, we demonstrate that the IKK complex additionally controls Relish transcriptional activity by direct phosphorylation. We have identified two residues on Relish, serines 528 and 529, which are phosphorylated by the IKK complex following immune stimulation. While these phosphorylation sites are not required for Relish cleavage, nuclear translocation, or DNA binding, they are critical for recruitment of RNA Polymerase II and antimicrobial peptide gene induction.

Introduction:

The *Drosophila* humoral immune response is characterized by the inducible expression of a battery of antimicrobial peptides (AMP). Following infection, the transcription of these genes is rapidly induced by NF-κB transcription factors. Two pathways control the activation of *Drosophila* Rel/NF-κB homologs and the induction of the AMP genes. The Toll pathway, which is stimulated by fungi and many Gram-positive bacteria, activates DIF and Dorsal, two p65-like proteins that are sequestered in the cytoplasm of quiescent cells by the one *Drosophila* IκB homolog, Cactus. Toll signaling leads to induction of antimicrobial peptide genes such as the antifungal *Drosomycin*. The IMD pathway, which is triggered by Gram-negative bacteria, activates Relish, a p100-like NF-κB precursor protein and results in induction of antimicrobial peptide genes including *Diptericin*.

In mammals, activation of the NF-κB proteins is regulated at multiple levels. Typically, in quiescent cells NF-κB proteins are sequestered in the cytoplasm through interactions with inhibitory IκB proteins, while NF-κB precursors, like p100 and p105, are held in the cytoplasm by their own C-terminal inhibitory IκB-like domain. A variety of inducers including microbial components lead to phosphorylation and ubiquitin-mediated degradation of IκBs, allowing nuclear translocation of NF-κB transcription factors. These stimuli also lead to direct phosphorylation of NF-κB proteins, which is linked to dimerization (Maier et al., 2003), nuclear translocation (Harris et al., 2006), DNA binding (Guan et al., 2005) or transcriptional activity (Yang et al., 2003; Zhong et al., 2002). Signal-dependent phosphorylation also plays an important role for regulation

of NF-κB precursors p100 and p105. For example, phosphorylation of p100 at C-terminal serine residues, by IKKα, is important for its signal-induced processing to p52 in the non-canonical NF-κB pathway (Amir et al., 2004; Liang et al., 2006).

Unlike the mammalian NF-κB precursors, Relish processing does not require the ubiquitin/proteasome pathway, but instead is an endoproteolytic, caspase-dependent event. Stimulation of the IMD pathway by Gram-negative bacteria (or DAP-type peptidoglycan (PGN)) leads to cleavage of Relish, producing an N-terminal RHD transcription factor module that translocates to the nucleus and activates immune genes, as well as a stable C-terminal domain that remains in the cytoplasm (Stöven et al., 2000). Relish cleavage occurs after residue D545, within a typical caspase target motif (LQHDG). DREDD, a *Drosophila* caspase-8 like protease, is required for Relish cleavage and physically interacts with Relish in cell culture. *Dredd* mutants do not induce antimicrobial peptide genes and are sensitive to gram-negative bacterial infections (Leulier et al., 2000; Stöven et al., 2003). Likewise, caspase inhibitors prevent Relish cleavage and AMP induction. Together, these data suggest that DREDD is the caspase that directly cleaves Relish, although this has not yet been demonstrated. It should also be noted that DREDD has been reported to function upstream in the IMD pathway, and is required for IKK activation, in addition to its proposed role in cleaving Relish (Zhou et al., 2005).

The *Drosophila* IKK complex is also necessary for Relish cleavage. The IKK complex contains two subunits: a catalytic kinase subunit encoded by *ird5* (IKKβ) and a regulatory subunit encoded by *kenny* (IKKγ) (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000). The activated IKK complex can directly phosphorylate Relish *in*

vitro (Silverman et al., 2000). Furthermore, *ird5* or *kenny* mutant larvae fail to cleave Relish, and targeting of the IKK by RNAi in cell lines also prevents PGN-induced cleavage and phosphorylation of Relish. Finally, the C-terminal 107 residues of Relish are required for both its phosphorylation *in vitro* and cleavage in cells (Stöven et al., 2003), suggesting phosphorylation and cleavage are linked, but a causal relationship has not been firmly established.

To date, the mechanisms involved in the control of Relish activation and cleavage by the IKK complex remain uncertain. In this report, we demonstrate that DREDD can directly cleave Relish and that the IKKβ directly phosphorylates two residues in the N-terminal transcription factor module of Relish, serines 528 and 529. However, these phosphorylation sites are not required for Relish cleavage, nuclear translocation or DNA binding. Instead, they are critical for the proper transcriptional activation of Relish target genes, via efficient recruitment of RNA Polymerase II to the promoters of antimicrobial peptide genes.

Results

We have previously demonstrated that 107 C-terminal residues of Relish are required for its IKKβ-mediated phosphorylation *in vitro* and signal-dependent cleavage in cells (Stöven et al., 2003). However, when all 10 serines and threonines in this 107 amino acid C-terminal region were changed to alanine, Relish was still phosphorylated normally *in vitro* and was cleaved in response to immune stimulation in cells (chapter II). Thus, the C-terminus of Relish is not a major target of IKK-mediated phosphorylation and phosphorylation of this region is not required for cleavage. Instead, this region is required for the interaction between Relish and IKKβ (chapter II).

Relish is Phosphorylated by IKKβ primarily on serine residue(s)

In order to begin to more thoroughly characterize the target(s) of IKKβ-mediated phosphorylation in a less-biased manner, we performed two-dimensional phosphoaminoacid analysis of *in vitro* phosphorylated Relish. Baculovirus expressed recombinant Relish was used as a substrate with recombinant IKKβ in a kinase assay. Phosphoaminoacid analysis of Relish revealed that IKKβ phosphorylates Relish primarily on serine residues, with a minor 6 % of phospho-Tyr and 8 % of phospho-Thr (Figure 3.1).

Immune-induced phosphorylation of Relish is dependent on the IKK complex and other IMD pathway components

To examine immune-induced phosphorylation of Relish, we generated two-dimensional phosphopeptide maps of *in vivo* labeled Relish, before and after PGN stimulation. FLAG-tagged Relish, stably expressed in S2* cells, was labeled with ^{32}P-orthophosphate. Cells were treated with caspase inhibitors, to prevent Relish cleavage, and then stimulated with PGN for 10 minutes, or left untreated. Relish was analyzed by 2D electrophoresis/chromatography. This analysis showed several phosphopeptides prior to immune stimulation, indicating that Relish is phosphorylated on multiple residues in a signal-independent manner. In addition, one phosphopeptide appears following PGN stimulation (arrow in Figure 3.2). In order to test if this signal-induced Relish phosphorylation is dependent on IKK complex, S2* cells were treated with dsRNA against IKKβ or IKKγ. RNAi treatment resulted in loss of the signal-dependent phosphopeptide spot (Fig. 3.2 A). Similarly, phosphopeptide maps of Relish from cells treated with RNAi against other IMD pathway components were missing the signal-induced phosphopeptide spot (Figure 3.2 B and C) while LacZ RNAi had no affect (data not shown). These results demonstrate that signal-induced phosphorylation of Relish requires DREDD, dIAP2, TAK1, TAB2, and the IKK complex, consistent with previous publications (Silverman et al., 2000; Vidal et al., 2001; Zhou et al., 2005).

Phosphorylation of serine 528 and 529

To identify the sites of signal-induced phosphorylation, mass spectrometry was performed on an uncleavable (D545A) form of Relish before and after PGN stimulation.

Relish was immunoprecipitated, separated by SDS-PAGE, stained with coomassie and excised. Sequence analysis was performed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS). These data identified several sites (S32, S35, S41, S65, S464-T469) of constitutive phosphorylation, but only two adjacent serines, 528 and 529, were phosphorylated in a PGN-dependent manner (Figure 3.3 A, data not shown). These two serines are just N-terminal to the cleavage site (D545), in the linker region of Relish, which separates the N-terminal RHD and the C-terminal ankyrin repeats.

To confirm the mass spectrometric data, 2D phosphopeptide maps of wild type and SS528/529AA mutant Relish were compared (Figure 3.3 B). The signal-induced phosphopeptide spot was not detected in SS528/529AA Relish, demonstrating that modification of serines 528 and 529 is responsible for the only signal-induced phosphorylation detected.

IKKβ directly phosphorylates serines 528 and 529

In order to determine if IKKβ directly phosphorylates serines 528 and 529, we performed *in vitro* kinase assays. Also, to more rapidly detect phosphorylation of serines 528 and 529, a phospho-specific antibody was generated. *In vitro* translated SS528/529AA mutant Relish exhibited thirty percent reduction in the level of IKKβ-mediated phosphorylation in a radiolabeled *in vitro* kinase assay (Figure 3.4 A). Furthermore, phosphospecific anti-Relish antibody recognized *in vitro* phosphorylated wild type Relish but not the SS528/529AA mutant, showing that these two serines are direct targets of IKKβ-mediated phosphorylation. Similarly in cells, wild type over-

expressed Relish, but not the SS528/529AA mutant, was detected with phosphospecific antibody after immune stimulation, demonstrating that serines 528 and 529 are also targets of signal-dependent phosphorylation in cells (Figure 3.4 B). A time course of PGN stimulation was performed to address the dynamics of phosphorylation and cleavage of endogenously expressed Relish in S2*cells. Endogenous Relish was cleaved and phosphorylated within 1 minute. Phosphorylation diminishes over time, such that is almost undetectable after 1 hour (Figure 3.4 C). Using the phosphospecific anti-Relish antibody, cleaved and phosphorylated N-terminal Relish was also detected in extracts from whole flies within 1 hour of infection (Figure 3.4 D).

Epistatic analysis of Relish phosphorylation

In order to analyze the epistatic relationship between Relish phosphorylation and the IMD pathway components, S2 cells expressing Flag-tagged Relish were treated with RNAi against most other members of the IMD pathway and cell lysates were analyzed with phosphospecific antibody. PGRP-LC, IMD, dIAP2, dFADD, DREDD, TAK1, TAB2, and IKKγ were all required for phosphorylation of Relish on serines 528 and 529 (Figure 3.5).

DREDD, but not IKK-mediated phosphorylation, controls Relish cleavage

In order to more fully characterize the mechanisms by which Relish cleavage is regulated, we generated stable cell lines that inducibly express the caspase-8 like DREDD, which has been proposed to be the Relish protease. Interestingly, expression of

DREDD leads to cleavage of Relish but not phosphorylation of Relish or *Diptericin* induction (Figure 3.6 A). Only catalytically active DREDD is able to induce Relish cleavage (data not shown). These results suggest that phosphorylation is not required for Relish cleavage.

Moreover, *in vitro* assays further support the notion that DREDD cleaves unphosphorylated Relish. Lysates from cells expressing either wild type or catalytically inactive DREDD were incubated with a biotinylated Relish peptide (Biotin-SGSGLQHD) and streptavidin beads were used to pull-down associated proteins. Wild type DREDD but not the catalytically inactive CA version bound to this peptide, as assayed by immunoblotting. This interaction was blocked by caspase inhibitors (Figure 3.6 B). *In vitro* cleavage assays were also performed with DREDD purified from these lysates with anti-epitope (V5) beads and recombinant Relish. In these assays wild type, but not catalytically inactive DREDD, was able to cleave recombinant Relish *in vitro*. This cleavage was also inhibited by caspase inhibitors (Figure 3.6 C). Phosphorylation of Relish, with recombinant IKKβ, did not improve the efficiency of this reaction (data not shown). These results demonstrate that DREDD can cleave Relish and forced expression of *Dredd* is sufficient to drive Relish cleavage. They also further argue that IKKβ-mediated phosphorylation is not involved in Relish cleavage.

Phosphorylation of Serines 528 and 529 is required for IMD signaling

Cells expressing wild type and SS528/529AA mutant Relish were analyzed for induction of antimicrobial peptide genes *Diptericin*, *Attacin* and *Cecropin*. In cell lines expressing the serine to alanine substituted Relish, induction of *Diptericin*, *Attacin* and

Cecropin genes is notably inhibited by this unphosphorylated version of Relish (Figure 3.7 A, left). In adult flies, induction of these antimicrobial peptide genes is completely abolished in Relish null flies. Transgenic expression of wild-type Relish in a null background can rescue lack of induction, whereas mutant transgenic Relish fails to rescue null phenotype (Figure 3.7 A, right). These results show that serines 528 and 529 are required for effective induction of antimicrobial peptides genes following stimulation of the IMD pathway. To determine how these serines function in the activation of Relish, we first examined signal dependent cleavage of Relish. Western blot analysis of stable cell lines that express wild-type or mutant Relish revealed that serines 528 and 529 are not required for signal-dependent cleavage of Relish. (Figure 3.7 B). Next, nuclear translocation of YFP-tagged Relish was observed in stable cell lines using confocal microscopy. Similar to wild-type Relish, the SS528/529AA mutant was mostly cytoplasmic in unstimulated cells and translocated to the nucleus upon PGN stimulation (Figure 3.7 C). To determine if phosphorylation of serines 528 and 529 control DNA binding, chromatin immunoprecipitation (ChIP) was used, with primers amplifying the region of the *Diptericin* promoter containing two well characterized κB binding sites (Georgel et al., 1993; Kappler et al., 1993; Meister et al., 1994; Reichhart et al., 1992). Both wild type and mutant Relish bound to DNA with equal efficiencies (Figure 3.7D), showing that serines 528 and 529 are not required for DNA binding either.

Efficient recruitment of RNA Polymerase II requires phosphorylation of serine 528 and 529

Our results demonstrate that phosphorylation of serines 528 and 529 regulate Relish activity downstream of DNA binding. In order to examine interaction of Relish

with other components of the transcriptional machinery, ChIP assay, with anti-RNA Polymerase II antibodies, was used. In particular, we compared recruitment of RNA Polymerase II to the *Diptericin* promoter in wild type and mutant cells. PCR analysis demonstrates that RNA Polymerase II was recruited to the *Diptericin* promoter more efficiently in wild-type Relish expressing cells than in mutant cells (Figure 3.8 B).

Discussion

To date, the mechanism(s) involved in the signal-dependent cleavage and activation of Relish remain uncertain. We have previously showed that the C-terminus of Relish is required for both its phosphorylation and cleavage (Stöven et al., 2003), suggesting phosphorylation might be required for cleavage. However, we have determined that the C-terminus is not the target of phosphorylation but is required for interaction between Relish and IKKβ (Chapter II). The IKK complex may control Relish cleavage independently of phosphorylation, which is consistent with a recent report, showing that TAK1 is not required for Relish cleavage (Delaney et al., 2006). However, TAK1 is required for IKK activation and, as shown here, Relish phosphorylation (Silverman et al., 2003).

In this study, we instead demonstrate that IKKβ-mediated phosphorylation controls signal-dependent Relish activation. Using mass spectrometry analysis and *in vitro* kinase assays, serines 528 and 529 were identified as direct targets of IKK phosphorylation. Phosphorylation of these residues is not required for signal-dependent Relish cleavage, nuclear translocation or DNA binding. Instead, ChIP experiments on *Diptericin* promoter demonstrated that they control efficient recruitment of RNA Polymerase II. The exact mechanism of how phosphorylation on serines 528 and 529 affect RNA Polymerase II recruitment needs to be elucidated.

In this report, we also demonstrate that DREDD can directly cleave Relish. Although DREDD is an initiator rather than an effector caspase, previous data strongly argued that DREDD is the caspase that cleaves Relish. Here we demonstrate that in cells,

overexpression of DREDD leads to cleavage, but not phosphorylation, of Relish. Furthermore, DREDD can directly cleave Relish *in vitro*. The control of Relish cleavage by a DREDD is an example of non-apoptotic functions for caspases. In mammals caspase 8 has also been implicated in numerous NF-κB pathways, including TLR signaling, but the molecular function of caspase-8 in these signaling pathways remains unresolved (Chun et al., 2002; Kang et al., 2004; Lemmers et al., 2007; Salmena et al., 2003).

Our data suggest that two distinct arms of the IMD Pathway control Relish activation. Upon immune stimulation Relish is endoproteolytically cleaved by the caspase DREDD. This cleavage requires Drosophila IKK complex but may not depend on its kinase activity. For the other arm of the pathway, the IKK complex phosphorylates Relish on serines 528 and 529 and this modification appears to be critical for the recruitment of RNA Polymerase II and transcriptional induction of AMP genes. However, IKK-mediate phosphorylation does not appear to be required for cleavage, nuclear translocation or DNA binding of Relish. Perhaps the interaction between Relish and certain co-activator(s) require phosphorylation of serines 528 and 529.

Materials and Methods:

Plasmids

Deletions and point mutations were generated by PCR-based site directed mutagenesis. FLAG-tagged WT and SS528/529AA mutant Relish were cloned into pCITE-2a(+) with T7 promoter for *in vitro* translation and pPacPL with actin promoter for cellular expression using standard methods. V5-tagged Dredd was cloned into pMT expression vector controlled by the metallothionein promoter.

Cell culture

Drosophila S2*cells were grown in Schneiders Media (Gibco) with 10% Fetal Bovine Serum, 1% Glut-MAX (Gibco) and 0.2% Penicillin-Streptomycin (Gibco) at 27°C. Cells were treated with 1μM 20-hydroxyecdysone for 24 hours before PGN stimulation (Invivogen).

dsRNA, DNA transfections and Stable Cell Lines

dsRNA was produced using T7 RiboMAX Express RNAi System (Promega). S2* cells were plated at a density of $1x10^6$ cells/ml. The cells were transfected with 1.5 mg/ml DNA or RNA using the calcium phosphate transfection method. 24 h later, the cells were split to $1x10^6$ cells/ml and treated with 20-hydroxyecdysone at 1μM for 24 h. Cells were then stimulated with peptidoglycan (Invivogen) for 5 hours for Northern Blots, up to 10 minutes for Relish cleavage and phosphorylation, and 15 minutes for nuclear

translocation and ChIP experiments. For stable cell lines, pPacPL constructs with actin promoter were transfected into Schneider S2* cells in conjunction with pHS-neo, 50:1; stable transfectants were then selected with G418 (800 μg/mL)**.**

RNA and Protein analysis

For Northern Blot analysis, total RNA was extracted with Trizol (Invitrogen), transferred to GeneScreen Plus Hybridization Transfer Membrane (Perkin Elmer) and blots were hybridized using the ExpressHyb (Clontech) system with radioactive probes. For Western Blot analysis, cells or flies were lysed in lysis buffer (20mM Tris pH 7.6, 150 mM NaCl, 25mM β-glycerophosphate, 2mM EDTA, 10% glycerol, 1% Triton X-100, 1mM DTT, 1mM $NaVO_4$, 1X Protease inhibitor cocktail, 100mM Okadaic acid), then total protein extracts were separated by SDS-PAGE and transferred to PVDF membrane. Antibodies used for immunoprecipitation or immunoblotting were as follows: anti-FLAG (Sigma), anti-V5 (Sigma). Phosphospecific antibody against Relish SS528529 was produced by immunizing rabbits with the Ac-FRKLIEHN(pS)(pS)DLEKIC-amide (520-535) phosphopeptide.

Kinase Assay

Versions of Relish were translated *in vitro* in reticulocyte lysates (Promega) and then immunoprecipitated by using anti-FLAG agarose (Sigma). 1/3 of these immunoprecipitates were used in the control Western blot, the rest in an *in vitro* kinase reaction with recombinant *Drosophila* IKKβ (Silverman, 2000) and γ-^{32}P-ATP. Kinase reactions were performed in kinase buffer (20 mM Hepes at pH 7.6, 20 mM beta -

Glycerolphosphate, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, 0.1 mM NaVO4, 200 μM ATP, and 5 μCi γ-^{32}P-ATP).

Phosphoaminoacid analysis

In vitro phosphorylated Relish was separated by SDS-PAGE, transferred to PVDF membrane, then excised and incubated with 6 N HCl at 110 °C for 1 h. The resulting amino acids were applied to thin-layer cellulose (TLC) plates with cold phosphoaminoacid standards. Phosphoaminoacids are separated from each other by electrophoresis in two dimensions: 20 min at 1.5 kV in pH 1.9 buffer (formic acid 25%, acetic acid 78%) followed by 16 min at 1.3 kV in pH 3.5 buffer (pyridine 0.5%, acetic acid 5%). Phosphoaminoacids were visualized by autoradiography and radioactive spots were aligned with cold phosphoaminoacid marker spots.

Two Dimensional Phosphopeptide Mapping

S2* stable cell lines that express wild type or mutant FLAG-tagged Relish were treated with 1mM ecdyson for 24 hours and radiolabeled with [^{32}P] orthophosphate for 8 hours in phosphate-free medium supplemented with 0.2 mM phosphate buffer. The cells were treated with 0.1 mM caspase inhibitor Z-VAD (OMe)-FMK (Calbiochem) for 20 min to block cleavage. After 10 min PGN stimulation, Relish was immunoprecipitated, resolved by SDS-PAGE and Western blotted. Relish is excised from PVDF membrane and digested with trypsin (Promega). The resulting peptides were resolved on thin-layer cellulose (TLC) plates (first dimension, thin-layer electrophoresis at pH 1.9; second dimension, thin-layer chromatography in n-butanol 37.5%, pyridine 25%, acetic acid 7.5%) and analyzed by autoradiography (Hardie, 1999).

Mass Spectrometry

The uncleavable form of the FLAG tagged Relish D545A mutant was expressed in stable cell lines. Relish is immunoprecipitated with anti-FLAG M2 agarose beads (Sigma), separated by SDS-PAGE and stained with coomassie. The Relish band is excised from the gel. Sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA XP Plus quadruPole ion trap mass spectrometer.

Confocal Microscopy.

Cell lines stably expressing YFP–Relish WT and SS528/529AA were generated by G418 selection. For confocal microscopy, stable cells were treated for 24 h with 20-hydroxyecdysone, then plated on concanavalin A–treated 35-mm glass-bottomed culture dishes and visualized by fluorescence microscopy with a 63X objective on a Leica SP2 AOBS laser-scanning microscope. Nuclei were stained with Hoechst 34580 (Invitrogen) and images were produced by sequential scanning with 514-nm laser excitation and a 522- to 599-nm emission window for YFP and 405 nm laser excitation and a 523- to 600-nm emission window for Hoechst 34580.

Binding Experiment for Active Dredd:

S2 cells were transfected with pMT expression constructs for either wild type or inactive (C408A), C-terminally V5-tagged Dredd and expression was induced with $CuSO_4$ for 15 h, in the presence or absence of zVAD-FMK (Sigma, 40 μM) or zLEHD-fmk

(Calbiochem, 50 μM). Cells were lysed in lysis buffer (50 mM Tris, pH7.5, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 10% Glycerol, 1mM DTT, 1 mM PMSF, 1 μg/ml Aprotinin). For the samples treated with zVAD/zLEHD, zVAD or zLEHD was also added to the lysis buffer. Lysates were incubated with 50 μM of Biotin-Relish-peptide (Biotin-SGSGLQHD-CHO) for 1h at RT. The formed caspase-peptide complexes were pulled-down with Streptavidin-Sepharose for 1h at 4C. Beads were washed with buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 5% Glycerol and boiled in loading buffer.

***in vitro* cleavage of Relish:**

Cells were transfected with *Dredd* wild type or C408A and lysed as above. Dredd-V5 was bound to V5-Agarose beads (Sigma), the beads were washed and incubated with 50 ng recombinant FLAG-Relish in 20 μl buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 5% Glycerol, 1 mM DTT) for 1h at RT. Beads were boiled in Loading buffer and the supernatant loaded onto and SDS-PAGE gel for Western Blot analysis.

Chromatin Immunoprecipitation

S2*cells and stable cell lines expressing Relish WT and SS528/529AA were treated with 1mM 20-hydroxyecdysone and stimulated with PGN for 15 minutes.

Cells were crosslinked with 1% formaldehyde for 10 min at room temperature and quenched with 120 mM Glycine. $5x10^6$ cells were used for each immunoprecipitation. Cells were harvested and spun at 700g for 5 min, washed 2 times with cold PBS, resuspended in 600 ml Sonication Buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5mM

EGTA, 1X Protease inhibitor cocktail (Sigma)) and sonicated 5 times for 10 sec with 1 minute intervals at 30% power using Fisher Scientific Model 500 Sonic Dismembrator. Sonicated lysates were spun at 8500g for 10 min and protein-DNA complexes were immunoprecipitated in IP buffer (0.1% SDS, 1% TritonX100, 0.1% Sodium Deoxycholate, 140mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM Tris 8.0, 1X Protease Inhibitor Complex, 1mg/ml BSA) overnight at 4ºC using 8WG16 (Covance) for RNA Polymerase II recruitment and anti-FLAG agarose (Sigma) for DNA binding. Beads were washed 3 times for 5 minutes with low salt buffer (0.1%SDS, 1% TritonX100, 0.1% Na Deoxycholate, 150 mM NaCl, 20mM Tris 8.1, 2mM EDTA, 1X Protease Inhibitor cocktail), 3 times for 5 minutes with high salt buffer (0.1%SDS, 1% TritonX100, 0.1% Na Deoxycholate, 500 mM NaCl, 20mM Tris 8.1, 2mM EDTA, 1X Protease Inhibitor cocktail), 2 times for 5 minutes with lithium buffer (0.25 M LiCl, 1% NP-40, 1% Na Deoxycholate, 10mM Tris 8.1, 1mM EDTA, 1X Protease Inhibitor cocktail) and 2 times for 5 minutes with TE. Protein-DNA complexes were eluted in 2x125 ml elution buffer (50mM $NaHCO_3$, 1%SDS). 15 ml of 5M NaCl was added to 250 ml of eluates to decrosslinked overnight at 65^{o}C. Eluates were column purified and analyzed by PCR.

Figure 3.1

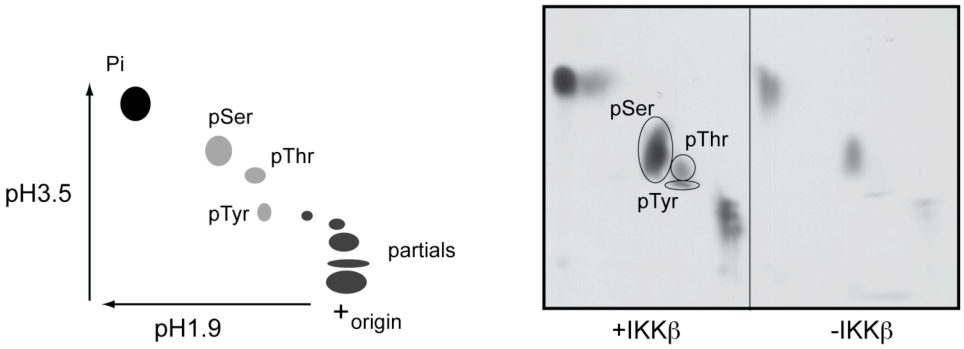

Pi
pSer
pThr
pTyr
partials
pH3.5
pH1.9
+origin
pSer
pThr
pTyr
+IKKβ
-IKKβ

Figure 3. 1 IKK-mediated phosphorylation of Relish is primarily on serine residues

Relish is phosphorylated *in vitro* by IKKβ and hydrolyzed with 6N HCl. The resulting aminoacids are separated by electrophoresis in two dimensions. The cartoon on the left demonstrates the positions of phosphoserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr) inorganic phosphate (P_i) and peptide products of partial digestion after acid hydrolysis.

Figure 3.2

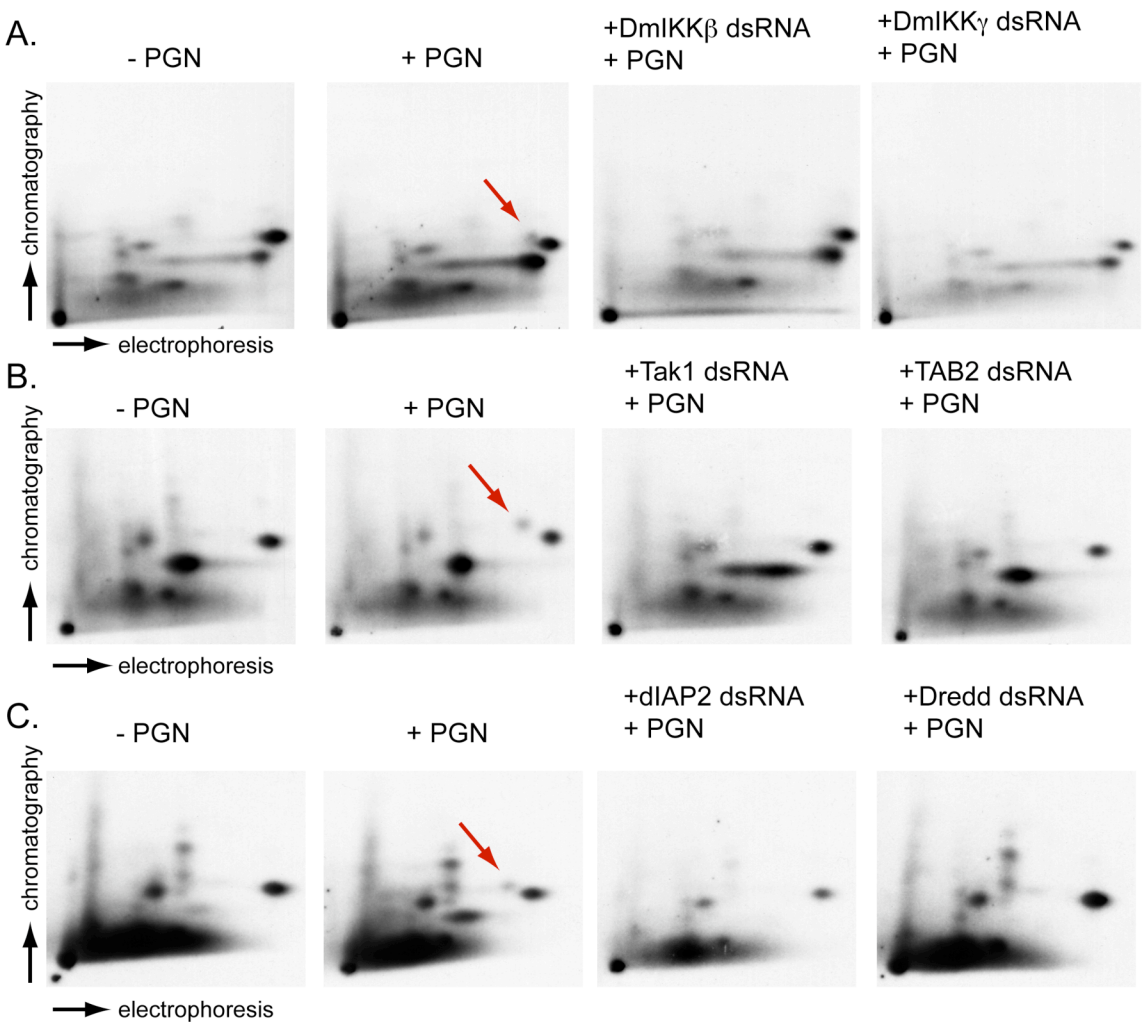
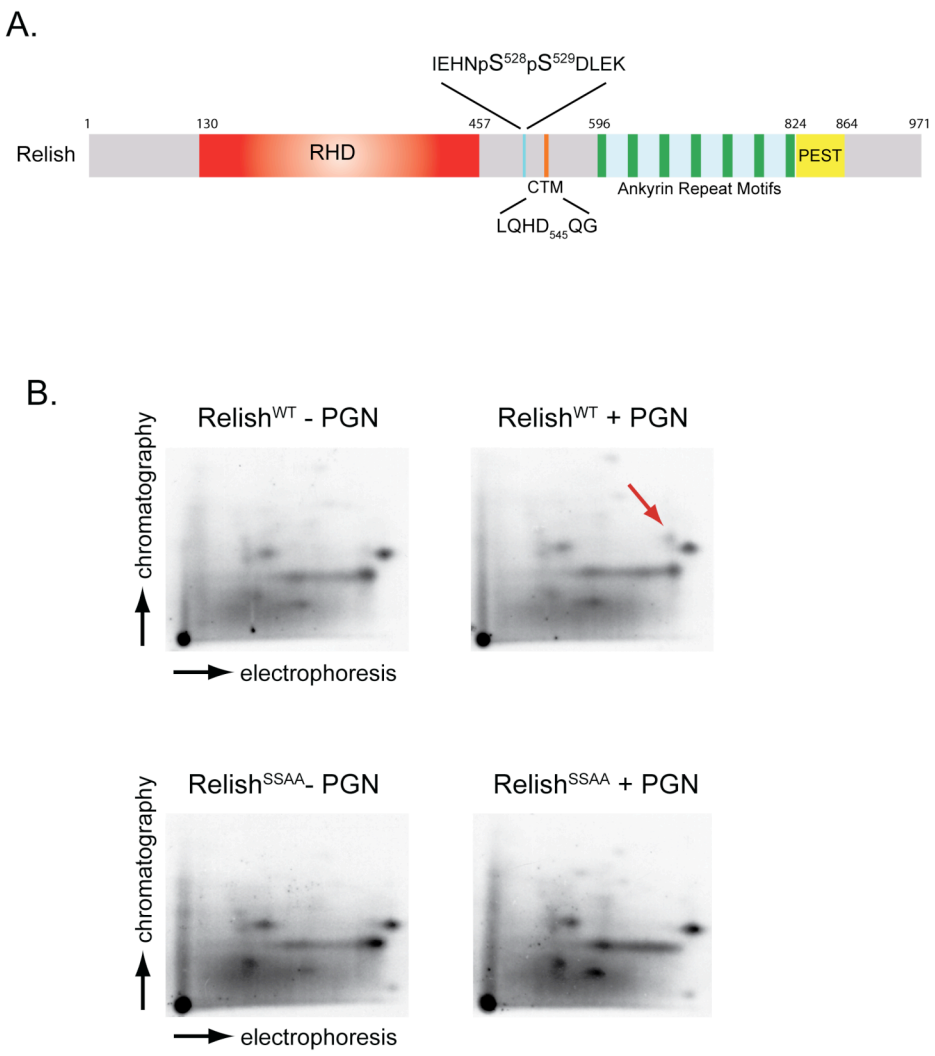

A.
- PGN
+ PGN
+DmIKKβ dsRNA
+ PGN
+DmIKKγ dsRNA
+ PGN
chromatography
electrophoresis
B.
- PGN
+ PGN
+Tak1 dsRNA
+ PGN
+TAB2 dsRNA
+ PGN
chromatography
electrophoresis
C.
- PGN
+ PGN
+dIAP2 dsRNA
+ PGN
+Dredd dsRNA
+ PGN
chromatography
electrophoresis

Figure 3. 2 2D phosphopeptide mapping of *in vivo* labeled Relish identifies a signal-induced phosphopeptide dependent on IMD pathway components

A. Signal dependent phosphorylation of Relish is mediated by *Drosophila* IKK complex. S2* stable cell lines that express wild type Relish were radiolabeled and treated with caspase inhibitor to block cleavage. Relish phosphopeptides were then analyzed by 2D phosphopeptide mapping. The arrow indicates the single phosphopeptide spot that reproducibly appears following peptidoglycan stimulation. Moreover, this phosphopeptide spot is not detected in cells treated with RNAi targeting DmIKKβ or DmIKKγ. **B** and **C**. Similar experiments were performed in cells treated with RNAi targeting other components of IMD pathway, TAK1, TAB2, dIAP2 and DREDD.

Figure 3.3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1
130
457
596
824
864
971
Relish
RHD
PEST
CTM
Ankyrin Repeat Motifs
LQHD545QG
B.
RelishWT - PGN
RelishWT + PGN
chromatography
electrophoresis
RelishSSAA- PGN
RelishSSAA + PGN
chromatography
electrophoresis

Figure 3. 3 Immune-induced phosphorylation of S528 and S529

A. Peptide sequence showing the phosphorylation and cleavage sites of Relish. Microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) identified serines 528 and 529 as the *in vivo* immune-induced phosphorylation sites on Relish. **B**. Serines 528 and 529 are phosphorylated *in vivo* in a signal dependent manner. Wild type and SS528/529AA mutant Relish were analyzed by 2D phosphopeptide mapping as in Figure 2. The phosphopeptide that appears upon peptidoglycan stimulation in wild type Relish is not detected in the mutant form of Relish.

Figure 3.4

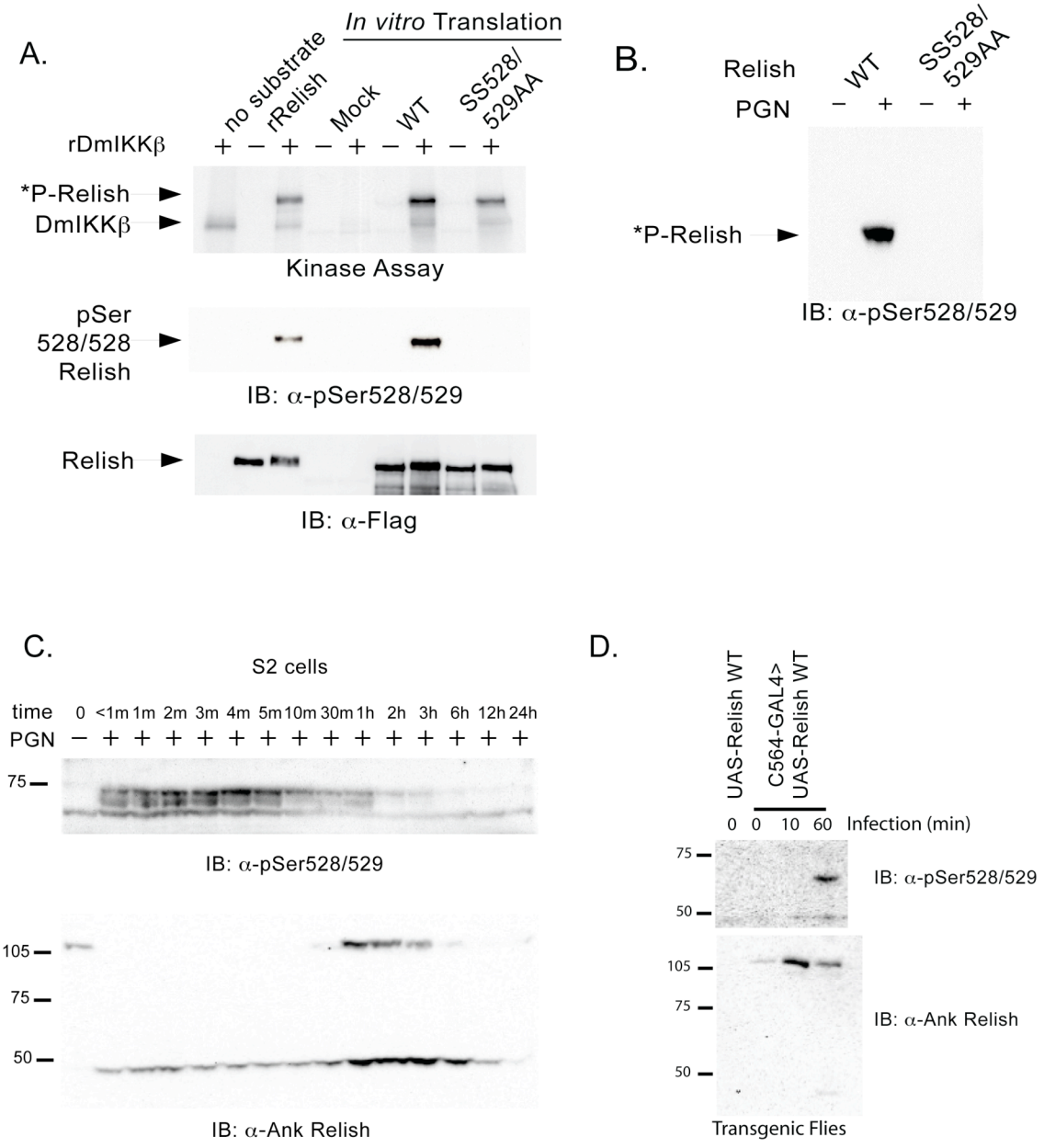

A.
In vitro Translation
no substrate
rRelish
Mock
WT
SS528/
529AA
rDmIKKβ
+ − + − + − + − +
*P-Relish
DmIKKβ
Kinase Assay
pSer
528/528
Relish
IB: α-pSer528/529
Relish
IB: α-Flag
B.
Relish
WT
SS528/
529AA
PGN
− + − +
*P-Relish
IB: α-pSer528/529
C.
S2 cells
time 0 <1m 1m 2m 3m 4m 5m 10m 30m 1h 2h 3h 6h 12h 24h
PGN − + + + + + + + + + + + + + +
75
IB: α-pSer528/529
105
75
50
IB: α-Ank Relish
D.
UAS-Relish WT
C564-GAL4>
UAS-Relish WT
0 0 10 60 Infection (min)
75
50
IB: α-pSer528/529
105
75
50
IB: α-Ank Relish
Transgenic Flies

Figure 3. 4 Characterization of anti-phospho-Relish antibody

A. Serines 528 and 529 are phosphorylated by IKKβ *in vitro.* Recombinant Relish or wild type and SS528/529AA mutant versions of Relish translated *in vitro* using rabbit reticulocyte lysate and immunoprecipitated with Flag-antibody, were used as a substrate in kinase reactions with recombinant *Drosophila* IKKβ and γ-^{32}P-ATP (top). 1/3 of each reaction was also immunoblotted with anti-phospho-Relish antibody, detecting serine 528/529 phosphorylation (middle) while the other 1/3 was immunoblotted with Flag antibody to confirm the presence of the Relish (lower). **B**. Serines 528 and 529 are phosphorylated in S2 cells. Lysates from stably transfected *Drosophila* S2 cells, expressing wild type or SS528/529AA mutant Relish, were analyzed. Immunoblotting using anti-phospho-Relish antibody detected serine 528/529 phosphorylated Relish. **C**. Endogenous Relish is phosphorylated in S2 cells rapidly following immune stimulation. **D** Serines 528 and 529 are phosphorylated following E. coli infection.

Figure 3.5

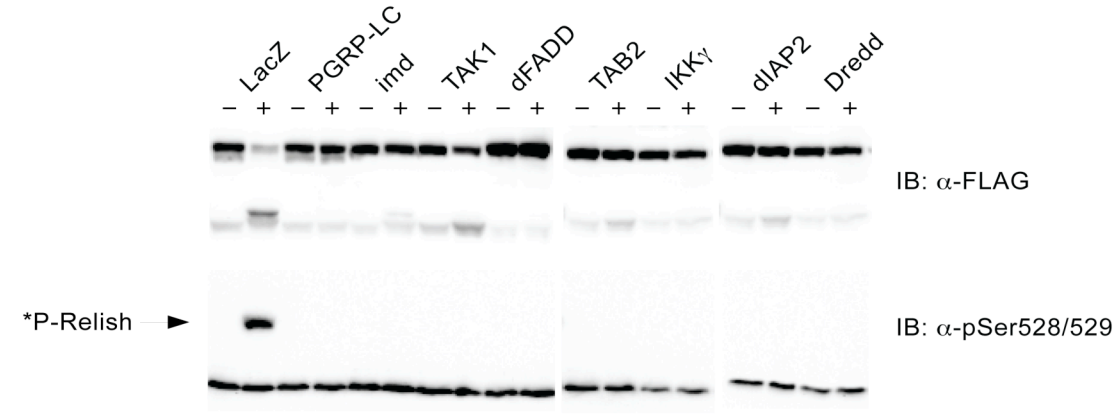

LacZ
PGRP-LC
imd
TAK1
dFADD
TAB2
IKKγ
dIAP2
Dredd
– + – + – + – + – + – + – + – + – +
IB: α-FLAG
*P-Relish
IB: α-pSer528/529

Figure 3. 5 Epistatic relationship between Relish and IMD pathway components

S2 cells expressing Flag-tagged Relish were treated with RNAi against IMD pathway components and cell lysates were analyzed with anti-FLAG antibody for Relish cleavage and phospho-specific antibody for Relish phosphorylation.

Figure 3.6

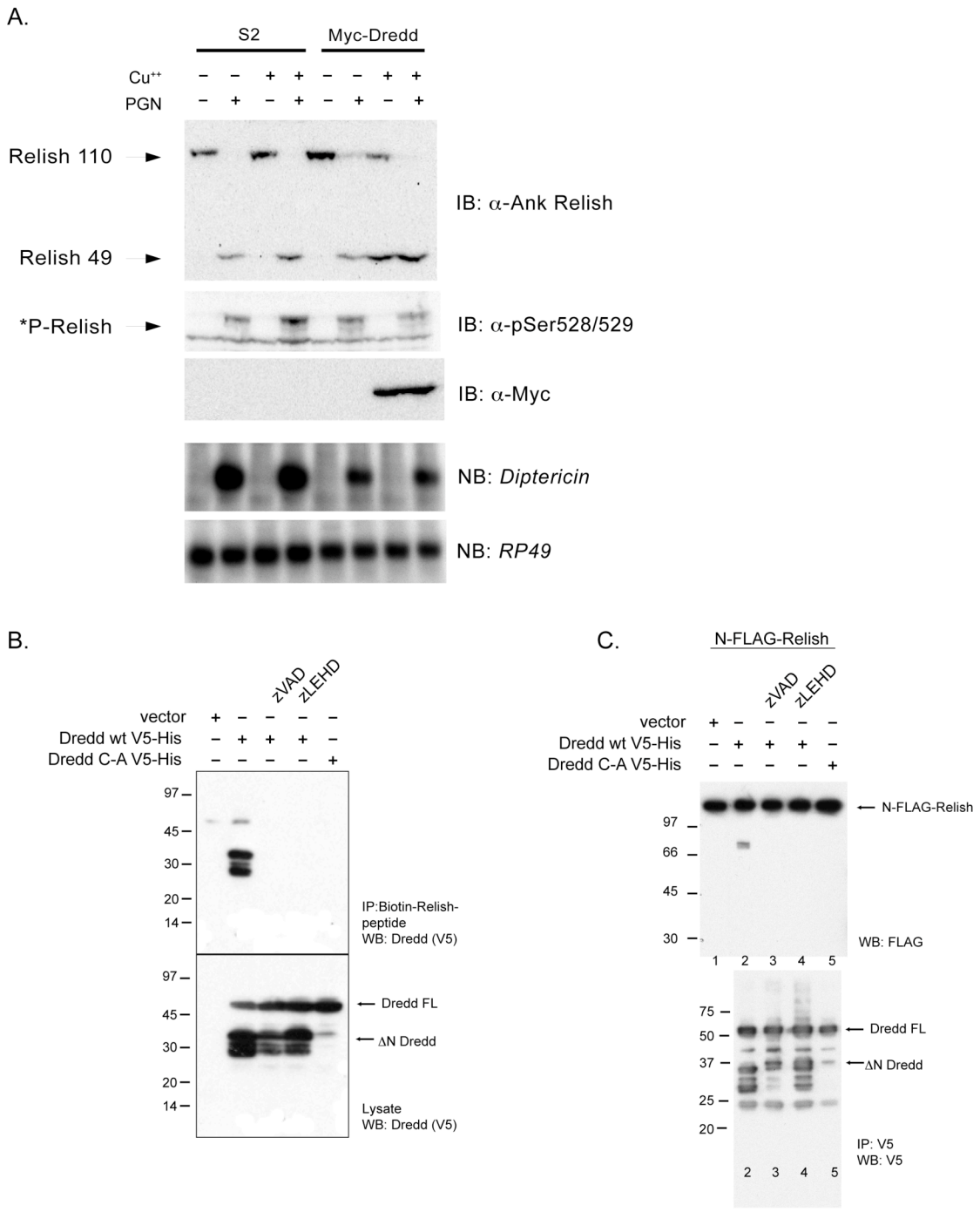

A.
S2
Myc-Dredd
Cu++
PGN
Relish 110
Relish 49
*P-Relish
IB: α-Ank Relish
IB: α-pSer528/529
IB: α-Myc
NB: Diptericin
NB: RP49
B.
zVAD
zLEHD
vector
Dredd wt V5-His
Dredd C-A V5-His
97
45
30
20
14
IP:Biotin-Relish-peptide
WB: Dredd (V5)
Dredd FL
ΔN Dredd
Lysate
WB: Dredd (V5)
C.
N-FLAG-Relish
N-FLAG-Relish
WB: FLAG
66
75
50
37
25
IP: V5
WB: V5

Figure 3. 6 The *Drosophila* caspase DREDD cleaves Relish

A. Overexpression of DREDD leads to Relish cleavage. Myc-tagged DREDD is overexpressed using Cu inducible promoter. Lysates were analyzed for relish cleavage and phosphorylation. Northern blot analysis was used to determine *Diptericin* expression. Overexpressed DREDD lead to Relish cleavage but not to its phosphorylation or Diptericin expression. **B**. Activated DREDD is pulled-down with a biotinylated Relish cleavage site peptide. pMT Dredd-V5 was expressed in S2 cells with or without caspase inhibitors zVAD-fmk and zLEHD-fmk. Wild type DREDD, but not catalytically inactive C-A is pulled down with Biotin-SGSGLQHD. The caspase inhibitors also prevent this interaction. **C**. The *Drosophila* caspase DREDD can directly cleave Relish *in vitro*. Active DREDD was isolated from S2 cell lysates with anti-V5 beads and incubated with recombinant FLAG-Relish. The N-terminal Relish product of cleavage is clearly detectable, by anti-FLAG immunoblot, with the wild type DREDD but not in the presence of caspase inhibitors or catalytically inactive DREDD-CA.

Figure 3.7

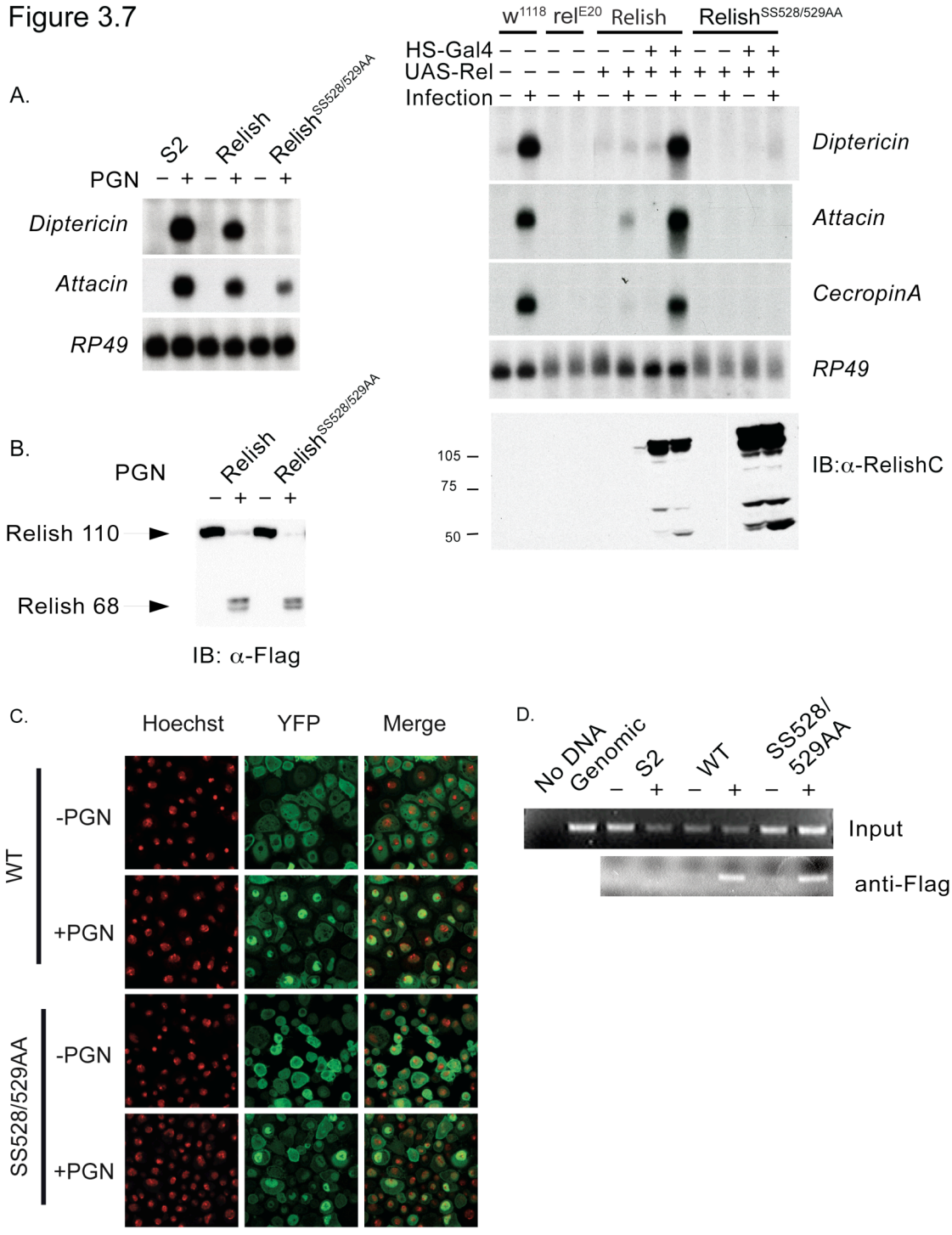
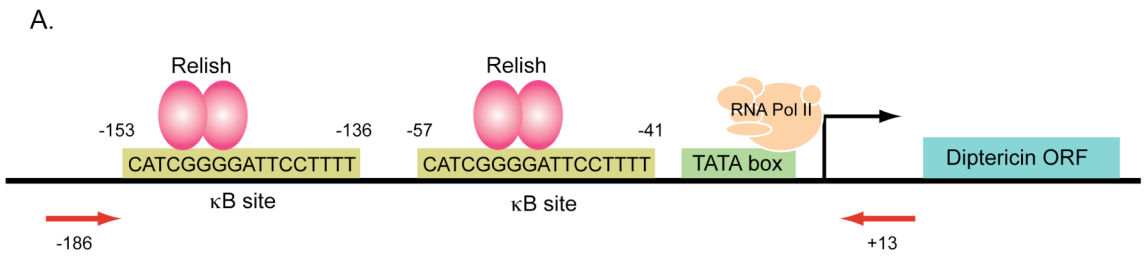

A.
S2
Relish
Relish^SS528/529AA
PGN – + – + – +
Diptericin
Attacin
RP49
B.
PGN
Relish
Relish^SS528/529AA
– + – +
Relish 110
Relish 68
IB: α-Flag
w^1118
rel^E20
Relish
Relish^SS528/529AA
HS-Gal4 – – – – – – + + – – + +
UAS-Rel – – – – + + + + + + + +
Infection – + – + – + – + – + – +
Diptericin
Attacin
CecropinA
RP49
105 –
75 –
50 –
IB:α-RelishC
C.
Hoechst
YFP
Merge
WT
-PGN
+PGN
SS528/529AA
-PGN
+PGN
D.
No DNA
Genomic
S2
WT
SS528/
529AA
– + – + – +
Input
anti-Flag

Figure 3. 7 Serines 528/529 are required for IMD signaling

A. SS528/529AA mutations block signal-dependent activation of antimicrobial peptide genes in cells and in flies. Cells stably expressing either mutant or wild type Relish were stimulated with E.coli PGN. Induction of antimicrobial genes was monitored by Northern Blotting with *Diptericin,* and *Attacin.* In cell lines expressing the SSAA mutant Relish, antimicrobial peptide gene induction was reduced (left). Transgenic flies expressing heat-shock driven mutant or a wild type Relish in null background were infected with E.coli. Lack of *Diptericin* and *Attacin* induction in Relish null flies (rel^{E20}) was rescued by expression of wild type transgenic copy, whereas the mutant copy fails to rescue antimicrobial gene induction (right). **B**. Serines 528 and 529 are cleaved in S2 cells. Lysates from stably transfected *Drosophila* S2 cells, expressing wild type or SS528/529AA mutant Relish, were analyzed. Immunoblotting using anti-FLAG antibody detected full-length and cleaved Relish. **C**. SS528/529AA mutant translocates normally to the nucleus. Nuclear translocation of wild type and mutant Relish proteins following peptidoglycan stimulation was analyzed by confocal microscopy. Mutant Relish was translocated similar to wild type, suggesting that those phosphoacceptor residues are not required for nuclear translocation of Relish. **D**. SS528/529AA mutant binds normally to DNA. ChIP assay on chromatin from S2* cells stably expressing wild type or SS528/529AA mutant versions of Relish using anti-FLAG antibody. Both wild type and mutant Relish bind to DNA normally.

Figure 3.8


A.
Relish
Relish
-153
-136
-57
-41
RNA Pol II
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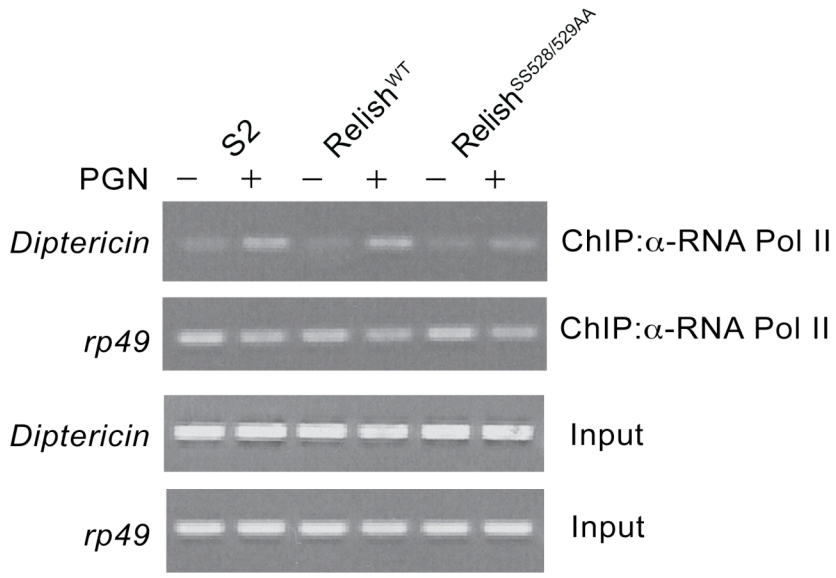
CATCGGGGATTCCTTTT
TATA box
Diptericin ORF
κB site
κB site
-186
+13


B.
S2
Relish^WT
Relish^SS528/529AA
PGN – + – + – +
Diptericin
ChIP:α-RNA Pol II
rp49
ChIP:α-RNA Pol II
Diptericin
Input
rp49
Input

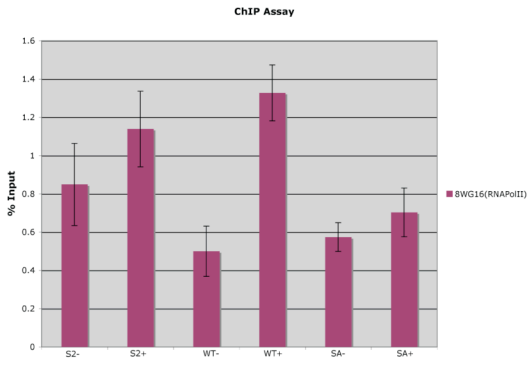

ChIP Assay
% Input
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
S2-
S2+
WT-
WT+
SA-
SA+
8WG16(RNAPolII)

Figure 3. 8 Recruitment of RNA Polymerase II to *Diptericin* Promoter

A. Schematic representation of the *Diptericin* promoter. The schematic represents the genomic DNA containing *Drosophila Diptericin* gene (not to scale). Upstream sequences contain two κB motifs where Relish can potentially bind. Red arrows indicate the primers used in ChIP assay. **B.** ChIP assay on chromatin from S2* cells stably expressing wild type or SS528/529AA mutant versions of Relish using monoclonal antibody against RNA Polymerase II (8WG16 from Covance), which recognizes the C-terminal heptapeptide repeat present on the largest subunit of RNA Polymerase II. Efficient recruitment of RNA Polymerase II requires phosphorylation of serines 528 and 529.

CHAPTER IV:

DISCUSSION

My thesis work has focused on understanding the signaling mechanisms in the *Drosophila* IMD pathway, in particular regulation of Relish activation by the *Drosophila* IκB Kinase complex. My studies have demonstrated that the IKK complex controls Relish activation by at least two distinct mechanisms: First, interaction of Relish with *Drosophila* IKKβ is required for its signal dependent cleavage. A death domain structure identified by sequence-structure homology prediction mediates this interaction. The second mechanism involves phosphorylation of serines 528 and 529 on Relish by the IKK complex following immune stimulation. These phosphorylation sites are not required for Relish cleavage, nuclear translocation or DNA binding. Furthermore, overexpressed DREDD can cleave Relish *in vitro* and in cells independent of phosphorylation suggesting that phosphorylation is not required for signal-dependent cleavage of Relish. On the other hand, phosphorylation of serines 528 and 529 are critical for efficient recruitment of RNA Polymerase II to promoters of antimicrobial peptide genes. Together these data describes a novel death domain in Relish and provides insights into the molecular mechanism of DREDD and IKKβ mediated Relish activation, which could lead to better understanding of insect immunity and similar innate immune signaling mechanisms in other organisms.

To date, the detailed mechanism of Relish activation and its regulation by the IKK complex has not been elucidated. Previous studies have shown that the IKK complex is required for signal-dependent cleavage of Relish and production of antimicrobial peptides, and that DmIKKβ can phosphorylate Relish *in vitro*. However, a link between phosphorylation and activation of Relish has not been established.

Phosphorylation plays an important role in regulating the functions of other NF-κB proteins (Drier et al., 1999; Guan et al., 2005; Maier et al., 2003; O'Shea and Perkins, 2008). In mammals, IKKβ phosphorylates inhibitors of NF-κB proteins; IκBα, β or ε at two serine residues in a conserved motif. This phosphorylation results in ubiquitination and proteasome-dependent degradation of IκB proteins. Furthermore, regulation of NF-κB precursors p100 and p105 also involves phosphorylation. In non-canonical NF-κB pathway, IKKα leads to phosphorylation of p100 at serine 866, 870 and 872 that triggers ubiquitination and subsequent processing of p100 to p52. Additionally IKKα phosphorylates p52 rel homology domain, which may play a role regulating other aspects of p52 function. Majority of p50 is constitutively and cotranslationally generated from p105. Inducible processing of p105 involves phosphoryations at serine 927 and 932 by IKKβ, which results in complete degredation of p105.

In contrast to mammalian NF-κB precursors, Relish processing does not depend on proteasome, instead relies on caspase activity. Other studies have shown that caspase-8 ortholog DREDD can interact with Relish and that it is required for signal-dependent Relish cleavage. However, biochemical evidence for a direct cleavage of Relish by DREDD has not been shown. This study provides evidence to answer these questions.

In the first chapter, the presented data described interaction between Relish and IKKβ demonstrating that the C-terminal 107 amino acids of Relish are required for its phosphorylation *in vitro* and its cleavage in cells suggesting phosphorylation and cleavage are related. However, a Relish mutant with all the phosphoacceptor sites mutated in this C-terminal region is still phosphorylated *in vitro* and cleaved in S2* cells normally, showing that Relish C-terminus is not a target of phosphorylation. Co-

immunoprecipitaion and yeast two-hybrid analyses demonstrated that C-terminus of Relish is required for its interaction with IKKβ.

Inspired by Aedes Relish, which is reported to have a C-terminal death domain I investigated the possibility of Relish having a death domain and its role in interaction with IKKβ. A similar mechanism is published showing that the death domain of mammalian NF-κB1 p105 is required for its interaction with IKK 1 and IKK 2 and is essential for signal-induced p105 proteolysis (Beinke et al., 2002). Using sequence-structure homology recognition program (FUGUE) we have modeled a predicted death domain in Relish C-terminus. Despite low-sequence homology, which is not uncommon in the death domain super family, Relish death domain structure fits the overall structural features of a death domain fold with six antiparallel α-helices forming a tightly packed hydrophobic core.

Small deletions in the C-terminal region containing the death domain or alanine substitution of a highly conserved tryptophan residue (W914A) block interaction of Relish with IKKβ suggesting an essential role for the death domain of Relish. Partial restoration of the interaction with a similar aromatic residue substitution (W914F) supports that idea. However, these mutations may be causing larger changes in the overall or local protein structure rather than affecting the IKKβ interaction surface. Further studies are needed in order to identify the interaction interface between Relish and IKKβ. The amino acids predicted to be on the surface of the death domain were identified (H905, D908, D911, E934, D935, K924 and R954). These residues need to be mutated and tested for interaction assays to verify their role in the interaction between Relish and IKKβ. The death domain is located at the extreme C terminus in most of the

death domain proteins and mediates homo or heterotypic interactions with other death domain family members. Interestingly, we found that the Relish death domain is required for interaction with IKKβ, which is not known to have a death domain. I predict that IKKβ also folds into a death domain-like structure and Relish and IKKβ interact through their death domains. Further studies are in progress to investigate this prediction.

The second chapter of this thesis focused on understanding the signal-dependent activation of Relish. Contrary to its mammalian counterparts p100 and p105, signal dependent processing of Relish is unique and requires caspase activity rather than the proteasome. We demonstrate for the first time that DREDD can directly cleave Relish. Despite being an initiator rather than an effector caspase, previous studies suggested that DREDD is the caspase that cleaves Relish. Here we demonstrate that in cells, overexpression of DREDD leads to cleavage, independent of phosphorylation. Furthermore, DREDD can directly cleave unphosphorylated Relish *in vitro* suggesting that phosphorylation is not required for cleavage, contrary to commonly accepted hypothesis that signal-dependent phosphorylation of Relish directs its caspase-dependent cleavage. The control of Relish cleavage by the caspase-8 homolog DREDD is an example of non-apoptotic functions for caspases. Similarly, in mammals caspase 8 has non-apoptotic roles in numerous NF-κB pathways, including TLR signaling, but the molecular mechanisms of caspase-8 in these signaling pathways remains unresolved. The relationship between Relish and DREDD could contribute to better understanding of non-apoptotic roles of caspases.

To date, the mechanism of how the IKK complex controls Relish activation and cleavage also remained uncertain. The data presented in this study demonstrates that

besides controlling Relish cleavage via interacting with the Relish death domain, IKKβ phosphorylates serines 528 and 529 in the N-terminal transcription factor module. However, these phosphorylation sites are not required for Relish cleavage, nuclear translocation or DNA binding. Instead, they are critical for the proper transcriptional activation of *Diptericin* by Relish, via efficient recruitment of RNA Polymerase II to the promoter region. The exact mechanism of how phosphorylations on serines 528 and 529 affect RNA Polymerase II recruitment and whether that mechanism controls activation of other target genes of Relish need to be elucidated.

One possible model is that the phosphorylation-dependent interaction of Relish with coactivators controls transcription of its target genes. I have tested possible coactivators such as CBP and Pcaf. CBP does not interact with Relish and Pcaf coimmunoprecipitates with both full-length and cleaved Relish independent of phosphorylation and immune signaling. Other candidates are mediator complex subunit Med16 and a recently identified member of IMD pathway called Akirin . Med16, a component of the multi subunit mediator complex, is shown to be required for immune induced gene activation. Mediator complex is known to allow specific transcription factors to communicate properly with Polymerase II and with the general transcription factors. Recently another member of the IMD pathway, named Akirin was identified and shown to be required for defense against Gram-negative bacteria. Akirin, which is strictly localized to nucleus and acts epistatically downstream of Relish is another candidate as an interacting cofactor. Further experiments are needed to investigate the interaction of Relish with Med16 and/or Akirin and whether phosphorylations at serines 528 and 529 are required for these interactions.

In summary, the data presented in this thesis elucidates the molecular mechanism of Relish activation, which involves two distinct signaling pathways downstream of the receptor PGRP-LC. One pathway controls the signal-dependent endoproteolytic cleavage of Relish by the caspase DREDD and requires IMD, FADD, and the IKK complex. DREDD is sufficient for Relish cleavage, and IMD and FADD most likely function to link DREDD to the receptor. However, Relish cleavage does not seem to be depending on the kinase activity of IKK complex. Instead, IKK complex may function as a scaffold or an adapter to facilitate the cleavage. Supporting this model, Relish interacts with IKK complex via its death domain and this association requires the very C-terminal domain of Relish, which is necessary for cleavage (Stöven et al., 2003) but is not the critical site of phosphorylation. The other pathway controls Relish phosphorylation through TAK1 and the IKK complex, which results in signal dependent phosphorylation of serines 528 and 529. However, these phosphorylations are not required for signal-dependent cleavage of Relish, nuclear translocation or DNA binding. These results are consistent with the findings of Delaney et al. (2003), who showed that TAK1 is not required for Relish cleavage. Other studies have demonstrated that TAK1 is necessary for the PGN-mediated activation of the *Drosophila* IKK complex (Silverman et al., 2003). This suggests that TAK1 is a critical component of the second arm of the IMD pathway, activating the IKK complex, which leads to phosphorylation of Relish on serines 528 and 529. This modification is critical for the recruitment of RNA Polymerase II and transcriptional induction of AMP genes. The interaction between Relish and certain co-activator(s), such as Akirin, might require phosphorylation of serines 528 and 529 (Figure 4.1)

Homologs of signaling pathway components involved in *Drosophila* innate immunity have been identified in humans and other organisms. In the absence of an adaptive immune response, studies in *Drosophila* provide an invaluable contribution to better understanding of the innate immunity in general. In that vein, this thesis explains the mechanism of Relish activation and its regulation by the IKK complex. These findings could help to answer other questions in the broader picture of innate immunity.

Figure 4.1

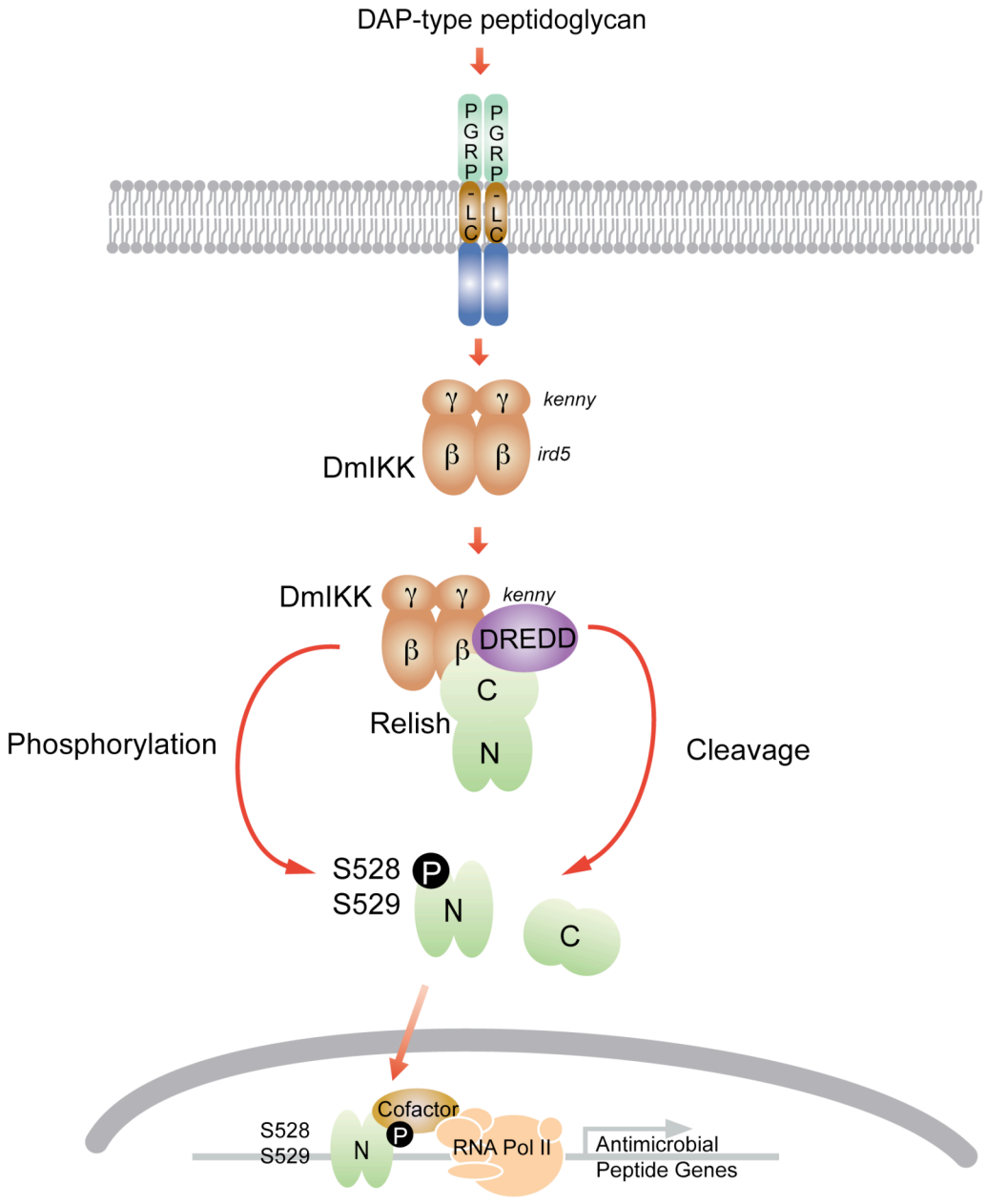

DAP-type peptidoglycan
PGRP-LC
PGRP-LC
γ
γ
kenny
DmIKK
β
β
ird5
DmIKK
γ
γ
kenny
β
β
DREDD
C
Relish
N
Phosphorylation
Cleavage
S528
S529
P
N
C
Cofactor
P
S528
S529
N
RNA Pol II
Antimicrobial
Peptide Genes

Figure 4. 1 Model for Relish activation

Drosophila IKK complex controls Relish activation by two distinct mechanisms. C-terminal 107 aminoacids of Relish contains a death domain, which is required for interaction between Relish and DmIKKβ. This interaction is necessary for signal-dependent cleavage of Relish suggesting that DmIKKβ may function as an adaptor to recruit DREDD to its target. On the other hand, we have identified two serine residues on Relish that are phosphorylated upon immune stimulation in an IKK-dependent manner. This phosphorylation is required for expression of antimicrobial peptides but not for cleavage, nuclear translocation or DNA binding of Relish. Instead, these phosphorylations appear to control efficient recruitment of RNA Polymerase II to the target genes. We predict that other coactivators, which remain to be identified, are recruited by phosphorylated N-terminal Relish transcription module.

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